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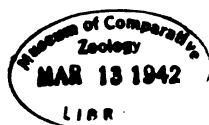
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OBSERVATIONS UPON THE MORPHOLOGY OF PARASITIC AND CULTURAL AMEBÆ.*

CHARLES F. CRAIG, M.D.

*(Captain, Medical Corps, U.S. Army.)**(From the Bacteriological Laboratory of the U.S. Army Medical School, Washington, D.C.)*

Our knowledge regarding the amebæ parasitic in man and their differentiation from free-living amebæ has been greatly extended since the work of Schaudinn,¹ published in 1903. Several new parasitic species have been described and it has been definitely proven by Hartmann, Viereck, Whitmore and others that the amebæ cultivated from the feces of dysenteric patients, as well as from the pus of liver abscesses, are in reality free-living species, having nothing whatever to do with the etiology of dysentery. In view of the interest attaching to this subject I have thought that a comparative morphological study of stained preparations of certain parasitic species and of a cultural ameba would be of value to workers in parasitology, and in the present paper the results are given of such a study of *Entameba coli*, *Entameba histolytica*, *Entameba tetragena*, and of a cultivated species which I have named *Ameba lobospinosa*.

Historical. — Casagrandi and Barbagallo,² in 1897, after a careful study of intestinal amebæ, reached the conclusion that they differed markedly in morphology and in life history from free-living, water amebæ, and established for them the

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genus *Entameba*. In 1903 Schaudinn³ accepted this genus for the parasitic amebæ of man and differentiated two species, a non-pathogenic species found in the intestine of a large proportion of healthy individuals, which he named *Entameba coli*, and a pathogenic species, found in the intestine of patients suffering from amebic dysentery, which he named *Entameba histolytica*.

While studying amebæ in soldiers invalided home from the Philippines at the U.S. Army General Hospital in San Francisco, I was able to confirm Schaudinn's results, and published my work in 1905,⁴ adding to it in another contribution published in 1908.⁵ Since the publication of my first paper the work of Schaudinn has been confirmed by Hartmann⁶ (1906-1908); Viereck⁷ (1907); Wenyon⁸ (1908); Werner⁹ (1909); Fantham¹⁰ and others, and the specific value of *Entameba coli* and *Entameba histolytica* definitely established.

In 1907, Viereck¹¹ described an ameba occurring in patients suffering from dysentery contracted in Africa, and during the same year an independent description of the same organism was published by Hartmann and Prowazek.¹² Viereck called the parasite *Entameba tetragena*, and as his publication appeared before that of Hartmann and Prowazek, who had named it *Entameba africana*, Viereck's name must be accepted as the proper zoölogical name of this species. These observations were soon confirmed by Bensen¹³ and by Werner,¹⁴ and in 1910 I was able to confirm the presence of this parasite in a soldier who had contracted dysentery in the Philippine Islands. In the publication¹⁵ relating to the demonstration of the new species in this case, I stated that *Entameba tetragena* is a frequent cause of dysentery in the Philippines, and shortly afterward Whitmore's important contribution¹⁶ upon parasitic and free-living amebæ appeared, in which the occurrence of *Entameba tetragena* in the Philippines was confirmed. He was unable to demonstrate *Entameba histolytica* in his material (only two cases are cited) and concludes that *Entameba tetragena* is the common cause of dysentery in Manila, *Entameba histolytica* being

seldom present. He was also able to confirm the occurrence of *Entameba coli* in the Philippine Islands.

In addition to these three well-defined species of parasitic amebæ, numerous other species have been described as occurring in man, as *Entameba tropicalis*, Lesage,¹⁷ 1908; *Entameba minuta*, Elmassian,¹⁸ 1909; and *Entameba nipponica*, Koidzumi,¹ 1909, but their specific value is still undecided and it is very probable that they all belong to one or the other of the three species already mentioned.

In regard to the position of cultural amebæ, it may be stated that there is no reliable evidence to date that any of those which have been cultivated, from any source, are identical with those parasitic in man. To Musgrave and Clegg²⁰ belongs the credit of first placing the cultivation of amebæ upon a practical basis, but unfortunately these investigators refused to accept Schaudinn's classification, and despite the fact that their cultivated amebæ contained a contractile vacuole (which is not present in any of the parasitic amebæ of man), and that the life cycle differed entirely from the parasitic species, they concluded that their cultural amebæ were identical with those concerned in the etiology of dysentery. Their conclusions in this respect were based largely upon the results of animal experiments, and these results were certainly very significant and will be considered later in this contribution.

Since the work of Musgrave and Clegg many investigators have confirmed the possibility of cultivating amebæ from various sources, but late researches have proven that all of the cultivated amebæ which have been carefully studied are free-living species which have contaminated the material from which the cultures were made. This subject has been very carefully investigated by Hartman and Werner, and in a recent paper by Whitmore²¹ the results are given of the study of cultures of amebæ from dysenteric feces and from the pus of a liver abscess, and the conclusion reached that all of the amebæ obtained in the cultures were free-living species having nothing in common with the amebæ occurring as true parasites in man. My results, to be given

later, confirm those of Whitmore, and I think that it may be stated as a fact that neither *Entameba coli*, *Entameba histolytica*, or *Entameba tetragena* has been artificially cultivated.

Material. — The material upon which the observations in this paper are based consists of the notes and drawings I have made during several years study of amebæ, and the recent study of two cases of infection with *Entameba tetragena*, contracted in the Philippines; one case of mixed infection with *Entameba histolytica* and *Entameba tetragena*, from Panama; three cases of infection with *Entameba histolytica*, from the Philippines, and three infections with *Entameba coli*. The cultural material consists of one culture obtained from Doctor Musgrave, of Manila, and two sent to this laboratory by the U.S. Army Board for the Study of Tropical Diseases, stationed in Manila, P.I.

In regard to the data drawn from my notes I would say that during my service at the U.S. Army General Hospital at San Francisco, I had the opportunity of studying the amebæ from one thousand and thirty-six cases of dysentery contracted in the Philippines, and careful records and drawings were made of the types of amebæ observed in these patients. A study of these records demonstrates that the majority of the infections were due to *Entameba histolytica*, but a considerable proportion were due to *Entameba tetragena*, which had not then been demonstrated as a distinct species. During my work in San Francisco I undoubtedly confused *Entameba tetragena* with both *Entameba histolytica* and *Entameba coli*, regarding organisms belonging to the former species as atypical examples of the latter, but there were hundreds of pure infections with *Entameba histolytica*, as proven by the morphology and life cycle of the amebæ present, and by the fact that the majority of the patients never showed the presence of the typical cystic forms of *Entameba coli* or *tetragena*, although observed for long periods of time, and under the most favorable circumstances.

Technic.—The amebæ were studied in both wet-fixed and air-dried preparations, stained with Dalafield's hematoxylin, Wright's modification of the Romanowsky stain, and Giemsa's stain. The wet-fixed preparations were fixed in sublimate alcohol as recommended by Schaudinn, and the technic of staining was that usually employed in the study of this class of organisms.²²

In my study of *Entameba coli* and *Entameba histolytica*,²³ published in 1908, I described the staining reactions of these parasites with the Wright method only, for the reason that the paper was intended as an aid to the practitioner, for whom it was thought the more complicated methods of staining, preceded by wet-fixation, would be unsuitable. As the method described in that contribution was sufficient for the differentiation of the two species of amebæ studied, and easy of application, it was hoped that thus a ready means of distinguishing between the harmless and pathogenic amebæ was available, but unfortunately some writers have concluded that my work is based entirely upon the study of preparations stained in this manner. This inference is incorrect as much of my material has been stained, after wet-fixation, with hematoxylin and other stains used in protozoölogical researches. In addition, my species differentiations are not only based upon properly stained specimens but also upon the morphology and life histories of the living amebæ, a most important and sadly neglected method of studying and differentiating these organisms.

However, it may be stated that preparations properly stained with the Wright or Giemsa stain are exceedingly useful in differentiating the parasitic amebæ, and the results obtained with these stains closely approach those obtained with wet-fixation and hematoxylin staining, although the latter methods give more uniform results and are more valuable in the study of morphological details. The results obtained in the study of the parasitic amebæ with Wright's or any other modification of the Romanowsky stain depend entirely upon technic. The stain must be carefully extracted, the amount of extraction being controlled by microscopic

examination, and if this is properly done similar results are obtained as regards morphology as those obtained with the other methods mentioned. Minchin,²⁴ in discussing the staining reactions of *Trypanosoma Lewisi* says: "That when the Romanowsky stain is cautiously extracted the nucleus comes down to a condition similar to that seen after other stains (iron hematoxylin, etc.); " and this statement is equally true of the staining reactions of the parasitic amebæ with the Wright or Giemsa modification.

In this paper I have described the morphology of wet-fixed preparations of the parasitic amebæ, stained with Delafield's hematoxylin, and, in addition, have noted for each organism the results obtained with the other methods. I believe that reference to the illustrations of the organisms described will show that there is not as much difference in the results obtained by the various methods as some authorities would have us believe. For the study of minute morphological details and the stages in the division of the nucleus of amebæ, wet-fixation, followed by staining with Delafield's or Heidenhain's iron hematoxylin should be used, but a great deal may be learned from specimens stained with Wright's or Giemsa's stains and the various species of parasitic amebæ may be well differentiated with these methods. Great care must be used in staining amebæ by any of the present methods if fine morphological details are to be studied, and even with the wet-fixation and hematoxylin methods the stain has to be very carefully extracted if good results are to be obtained.

In the study of cultural amebæ I have found that only the wet-fixed, hematoxylin preparations are of service, as the karyosome of the nucleus in these amebæ is so large and the amount of chromatin so great, that it is impossible to secure differentiation of the nuclear structures with either the Wright or Giemsa stain. With the latter the entire nucleus appears as a spherical, deeply-stained body without definite morphological details.

The morphology of amebæ in stained preparations varies

at different stages in their life cycle, and in this paper the morphology of the vegetative stage of development will first be described, followed by that of the encysted stage.

ENTAMEBA COLI. (Loesch, 1875; Emend Schaudinn, 1903.)

This ameba is found in the intestine of normal individuals in all parts of the world, the percentage of infection varying from five to seventy in different localities. The organism is non-pathogenic and is chiefly of interest because it is so often confused with the pathogenic amebæ causing dysentery.

Vegetative stage of development. — During this stage the size of the parasite varies considerably in stained specimens, measurements of from five to fifty microns in diameter having been recorded, but the size generally averages fifteen to twenty-five microns in the wet-fixed preparations.

There is no distinction between the ectoplasm and the endoplasm in specimens stained with hematoxylin, but in those stained with the Wright or Giemsa stain the ectoplasm takes a light blue color while the endoplasm stains a darker blue and appears much more granular. The vacuoles show much better in the Wright stained preparations, and appear more numerous. The endoplasm often contains crystals and numerous bacteria and very rarely a red blood corpuscle may be observed. The bacteria are more readily distinguished in specimens stained with one of the modifications of the Romanowsky method, as they take a deep red or purple color, while the cytoplasm of the ameba is stained blue. The stain should be well extracted, especially in hematoxylin preparations, if the structure of the cytoplasm is to be distinguished.

The nucleus of *Entameba coli* is quite characteristic and varies considerably in appearance at different stages in the life cycle of the organism.

In preparations wet-fixed and stained with hematoxylin the nucleus takes a dark purple color, and when the stain has been well extracted with alcohol or acid alcohol the structure is beautifully shown.

Before division the nucleus measures from five to eight microns in diameter and shows a well-marked, intensely stained karyosome, situated at or near the center, which, in some instances, contains a minute centriole. Surrounding the karyosome may sometimes be seen an unstained halo and lying between the latter and the nuclear membrane minute granules or masses of chromatin may not infrequently be observed, but these are generally small in number and are often absent. The nuclear membrane stains well, is generally of considerable thickness, and situated upon its inner side are hemispherical or flattened masses of chromatin, which may be arranged regularly or irregularly around the membrane. In some instances the membrane is broken into deeply-stained rod-like masses or dots of chromatin, separated by unstained intervals.

The nucleus varies much in appearance in different amebæ and at different times, due perhaps to cyclical changes taking place within it, or to the staining process, but one is always able to distinguish the karyosome and the nuclear membrane with its masses of chromatin, during the period before the division of the nucleus. Reference to the illustrations will show some of the variations which are most frequently observed (Plate I.).

In the small or young amebæ the nucleus is a spherical body having a well defined nuclear membrane and a very minute karyosome. In such amebæ the nuclear membrane appears smooth, no masses of chromatin being situated upon the inner surface, nor can free chromatin be demonstrated within the nucleus.

During the vegetative stage of development *Entameba coli* reproduces by simple division and by schizogony, the latter process giving rise to eight daughter amebæ.

In organisms undergoing simple division the nucleus shows evidences of a primitive form of mitosis, but I have never observed the formation of an equatorial plate or a typical nuclear spindle in this species, although such appearances are commonly found in stained specimens of cultural amebæ. Division is first evidenced in the karyosome, which

may sometimes be seen divided into two portions connected by a delicate filament of chromatic substance. In those organisms in which simple division of the nucleus is well advanced the nucleus is elongated, the karyosome has disappeared, and two deeply stained polar bodies are present, which may be connected by a thread of chromatin or a little amorphous granular material may lie between them, the remains of the chromatin thread originally connecting the dividing portions of the karyosome. When nuclear division is complete two nuclei may be observed within the body of the parasite, each having a thin nuclear membrane and a small karyosome. At this stage of development the nucleus is quite similar to that of *Entameba histolytica*, but the latter is much more delicate in appearance, the nuclear membrane being much thinner, while the karyosome is often absent or consists of a very minute dot or granule.

The cytoplasm of the parasite does not present any distinctive features, in stained specimens, during simple division of the nucleus.

In organisms undergoing schizogony, a reproductive process very seldom observed in my experience, the nucleus appears much swollen while the amount of chromatin within it and upon the nuclear membrane is greatly increased, the karyosome being invisible, in most instances. These changes occur at the beginning of the process and are followed by the collection of the chromatin in eight hemispherical masses situated within the nucleus or arranged upon the inner side of the nuclear membrane, in a very regular and characteristic manner. At this stage of development the chromatin stains very intensely and the arrangement of the chromatin masses can be easily studied.

Eventually the nuclear membrane disappears and specimens are observed in which the eight small masses of chromatin are free within the cytoplasm of the parasite. A nuclear membrane surrounds the chromatin masses and thus the daughter nuclei are formed. When fully developed each nucleus has a well-marked nuclear membrane and a small karyosome, and they are arranged somewhat regularly

within the cytoplasm of the parasite. At this stage of reproduction *Entameba coli* might again be mistaken for *Entameba histolytica*, the daughter nuclei being confused with the chromidial masses found in the cytoplasm of the latter organism, but the uniform presence of eight such masses and their nuclear structure should be sufficient to prevent such an error. The young amebæ produced by schizogony stain deeply and have the same nuclear structure as those produced by simple division.

Cystic stage of development. — When conditions are unfavorable for the vegetative existence of *Entameba coli*, the organism encysts and reproduction occurs within the cyst. Thus, in this species of ameba encystment is not only a protective process but also a reproductive one.

The cysts measure from ten to twenty microns in diameter and have a distinct wall which may present a single or double outline, in fresh preparations, and which is generally smooth but may be slightly mammillated. The cytoplasm stains homogeneously or presents a slightly granular appearance, while the nucleus or daughter nuclei are clearly defined in well stained preparations. The cytoplasm sometimes contains one or two irregular masses of chromatin, staining intensely, and very similar in appearance to those occurring in the cysts of *Entameba tetragena* which are so characteristic of the cysts of the latter organism. Werner²⁸ has also described large chromatin masses in the cyst of *Entameba coli*, but in my experience these masses are much more irregular in shape and smaller in size than those characteristic of *Entameba tetragena*. Frequently the cytoplasm is practically unstained.

The cytoplasm may also contain rods, filaments, and granules of chromatin in those organisms in which the nucleus is not visible, and one or more vacuoles may sometimes be observed, while in cysts which are undergoing degeneration the entire cytoplasm may be filled with vacuoles, the nucleus having disappeared.

Very complicated nuclear changes occur during reproduction within the cyst and these were carefully worked out by

Schaudinn,²⁶ and have been confirmed by numerous authorities. In stained preparations it is very difficult to be sure of the various steps in the process of reproduction, but the following morphological details may be observed in wet-fixed specimens, stained with Delafield's hematoxylin.

Evidences of the primary division of the nucleus within the cyst are frequently observed and the process is identical, so far as I have been able to determine, with that occurring during simple division, the karyosome first dividing, followed by the appearance of polar bodies in the nucleus, and finally by the division of the nucleus into two, the morphological picture being the same as that described in the consideration of simple division.

The morphology of the process of autogamy, which occurs after the primary division of the nucleus, is obscure, but cysts are frequently observed which contain from one to eight nuclei, and I have seen a few in which as many as ten and twelve nuclei could be counted. The most common numbers present are one, two, four, six, or eight, and when the process of nuclear division is completed the cysts of *Entameba coli* always contain eight daughter nuclei. The parasites showing more or less than eight nuclei are passing through the various stages in the process of reproduction and it will be found that the eight-nucleated cyst is most frequently encountered and is characteristic of this species of ameba.

In well stained specimens the daughter nuclei are spherical in shape, have a distinct nuclear membrane, which frequently appears much thickened at some portion of the periphery, while a distinct karyosome may or may not be present. Generally the karyosome is represented by a few granules of chromatin loosely arranged near the center of the organism, and dots and grains of the same material may be observed scattered throughout the nucleus, or collected in minute masses upon the inner side of the nuclear membrane. The thickening of the membrane at one portion of the periphery appears to be due to the concentration of the

chromatin, and is quite frequently observed in the cysts showing from four to six nuclei.

Cysts are also observed in which the nucleus cannot be distinguished, but the cytoplasm contains many small masses of chromatin or the entire cyst is filled with deeply stained granules of this substance of uniform size. These correspond to the cysts described by Schaudinn, in which the nucleus liberates its chromatin to the cytoplasm and the daughter nuclei are developed from the free chromatin. Such cysts always have a thick membrane which may appear mammillated.

A primitive mitosis may be demonstrated during the division of the daughter nuclei, consisting in the presence of deeply staining polar bodies connected by more or less chromatic material, but mitosis is never so typical as it is in the nucleus of cultural amebæ. In fact, the division of the nucleus in *Entameba coli*, during every stage of its development, differs markedly from that present in any cultural ameba I have studied, and this is also true of nuclear division in the other parasitic amebæ of man.

Degenerative forms of *Entameba coli* are frequently encountered in stained preparations, both during the vegetative and encysted stages of development, and have often lead to confusion in the differentiation of the species. In such organisms the nucleus may be invisible or devoid of a karyosome, while no morphological details can be distinguished because of the diffuse staining of the nuclear substance. The endoplasm may be filled with vacuoles and free chromatin may be present, derived from the degenerated nucleus. In the cystic stage the entire cyst may be vacuolated, the daughter nuclei absent or deformed, while irregular masses of chromatin may be scattered throughout the cytoplasm. These degenerative forms have in the past led to the description of new species and to contradictions in the description of *Entameba coli* by different observers.

The staining reactions of this parasite with the Wright or Giemsa stain are very similar to those obtained with

wet-fixation, followed by hematoxylin, especially if the specimens are first fixed with osmic acid vapor or sublimate alcohol. I am fully in accord with the conclusion of Wasielewski²⁷ that specimens so fixed and then stained with the Romanowsky stain or its modifications give most beautiful pictures of the morphology of this class of parasites, but the results cannot be said to be as uniform and greater care is needed in securing good specimens; so that for morphological studies of the amebæ the use of either Delafield's hematoxylin or Heidenhain's iron-hematoxylin is preferable. Unless the stain is very carefully extracted after the use of Wright or Giemsa's solutions, minute morphological details are hidden by the excessive precipitation of the chromatin stain. The nucleus always appears larger when these stains are used, as does the karyosome, which frequently, in poorly washed specimens, fills the entire nucleus, thus resembling the karyosome of cultural amebæ. For species differentiation these stains are very useful, however, and the poor results reported by some observers have been due to imperfect staining solutions or to technical errors. The study of the morphology and life cycle of this organism in living specimens should never be neglected as the results so obtained are even better than those arrived at from the study of stained preparations.

ENTAMEBA HISTOLYTICA. (Schaudinn, 1903.)

This is a pathogenic species of ameba, the cause of a form of dysentery especially prevalent in tropical and sub-tropical countries. The relative frequency of this species and of *Entameba tetragena* has not, as yet, been determined, but in my experience the majority of the dysentery patients who contracted the disease in the Philippines between 1899 and 1905 were infected with this parasite. *Entameba histolytica* has been demonstrated in the Philippine Islands, Formosa, Cochin China, Siam, India, Africa, South America, Panama, and the United States.

The morphology of the parasite, in common with all

amebæ, varies greatly at different stages in the life cycle. The methods of reproduction may be studied in both the living and stained organisms, but many preparations must be examined before every step in the reproductive processes can be demonstrated. It is also necessary to examine preparations from patients in various stages of the disease as the acute cases show only the vegetative organisms, the sporulating stage of development being found only in patients in whom the disease is chronic or who are recovering from the infection. Even in the chronic cases the process of sporulation is observed only during periods of improvement, never when the symptoms are acute.

Vegetative stage of development. — In preparations fixed with sublimate alcohol and stained with Delafield's hematoxylin the morphology of this species of ameba is well demonstrated.

In such specimens the size of the organism varies from fifteen to sixty microns in diameter, the average being from twenty-five to fifty microns. The shape is usually spherical or roughly oval, but is sometimes very irregular. There is no distinction between the ectoplasm and endoplasm, the entire cytoplasm appearing coarsely granular, and it may contain one or more vacuoles, crystals and bacteria, and red blood corpuscles if the feces contain blood. Not infrequently the cytoplasm is almost filled with erythrocytes, the nucleus and vacuoles being crowded to the periphery of the organism. Small unstained, or dimly stained, oval or round areas are frequently observed in the cytoplasm and, during certain stages of development, innumerable grains, threads, or clumps of chromatin are noted throughout the parasite. Vacuoles are almost always present, the only exceptions being the very small, or young amebæ, and are generally multiple in number, frequently containing amorphous material, thus indicating their digestive nature.

The nucleus is usually invisible in the living parasite but can be well differentiated in stained preparations. It varies considerably in size, measurements of from three to eight microns having been recorded, but in my experience the

average diameter of the nucleus has been about five microns. The nucleus is spherical or oval in shape and is situated excentrically, being found near the periphery of the organism or even flattened out against the limiting membrane of the parasite. In the living organism the nucleus does not appear to possess a limiting membrane, but in well stained preparations a delicate, deeply stained nuclear membrane, not over a line in thickness, can be distinguished. Upon the inner side of this membrane very minute granules of chromatin may sometimes be distinguished, while a very small and delicate karyosome may be observed near the center of the nucleus, usually composed of a single dot or small mass of chromatic substance. I have never been able to demonstrate the presence of a centriole in the karyosome of this species.

Lying in the hyaloplasm between the karyosome and the nuclear membrane may sometimes be seen a few minute grains of chromatin and traces of a linin network, but many organisms are observed in which the nuclear structure consists only of a delicate nuclear membrane enclosing the minute karyosome surrounded by an unstained substance. The amount of nuclear chromatin at this stage of development of *Entameba histolytica* is much less than in either *Entameba coli* or *Entameba tetragena* and the entire nucleus is small and more delicate in appearance.

Reproduction during the vegetative stage occurs by simple division and, I believe, by a process of budding or gemmation, similar to the process of sporulation or cyst-formation to be described later, but in which no resistant spores or cysts are developed.

In simple division the nucleus divides by a primitive mitosis, the karyosome separating into two very minute dots connected by a delicate chromatic thread, the nucleus at the same time becoming elongated. When the process is further advanced traces of a nuclear spindle may be observed in some organisms, but I have never seen the beautiful mitotic figures often observed in cultural amebæ. Rarely an organism may be observed containing two nuclei, each having a

delicate nuclear membrane and a minute karyosome. After the division of the nucleus the cytoplasm divides into two portions, each containing one of the nuclei, and thus two amebæ are produced.

In stained preparations from acute cases of dysentery due to *Entameba histolytica* organisms are sometimes observed in which the nucleus has disappeared and the cytoplasm is filled with irregular masses of chromatin, which, in some instances, are mostly collected at the periphery of the parasite, and are apparently being extruded, surrounded by a portion of cytoplasm. I believe that such organisms are undergoing a process of reproduction analogous to the schizogony of *Entameba coli*, and only differing from true sporulation in that resistant spores or cysts are not produced, each bud, as it may be called, immediately becoming a vegetative ameba. In the living specimen I have seen these bodies, after being pinched off from the periphery of the ameba, develop ameboid motion and move away from the parent body. The process is not degenerative in character, as shown by the uniformity in size of the bodies (which is not true of fragments produced by the fragmentation of the organisms) and by the development of ameboid motion and the presence of a nucleus in the daughter amebæ.

Sporulating or cystic stage of development. — In the feces of dysentery patients who are recovering from the infection forms of *Entameba histolytica* are observed which are never found during the acute stage of the disease or when acute symptoms are present in the more chronic cases. These forms, which Schaudinn first described and which he called "sporulating forms," are characteristic of this species and may be well studied in either fresh or stained preparations. They result in the formation of small resistant spores or cysts, which, under favorable conditions, become vegetative parasites and which are capable, as proven by Schaudinn, of producing typical amebic dysentery.

The process of reproduction by cyst formation in this species, stated briefly, consists in the distribution of the

nuclear chromatin to the cytoplasm, where it apparently multiplies, collects at the periphery of the organism, and is budded off, surrounded by some of the cytoplasm, after which each little mass (which is thus composed of chromatin and cytoplasm) becomes surrounded by a resistant cystic membrane. Organisms illustrating every stage of this process may be observed in both fresh and well stained preparations.

In specimens fixed in sublimate alcohol and stained with hematoxylin the first evidence of the process is observed in the nucleus, which appears swollen and much richer in chromatin. The nuclear membrane, instead of being represented by a delicate line of stained material, is broken and contains granules and threads of chromatin, while the karyosome has disappeared, the hyaloplasm containing much loose chromatic material. At a little later stage in the process the nuclear membrane has disappeared in places and the cytoplasm in the vicinity of the nucleus contains threads and granules of chromatin. Still later the nucleus stains very poorly, is free from chromatin, and is crowded against the periphery of the parasite, appearing as a flattened disc without definite structure; the cytoplasm is filled with rods and threads of chromatin distributed uniformly throughout it or collected in minute, loosely arranged masses, most numerous toward the periphery of the parasite. In a still more advanced stage of the process the chromatin is found distributed around the periphery, being concentrated in certain areas, thus causing a bulging of the periphery. The final stage is observed in the parasites showing appearances like those pictured in Plates I. (II.), and II. (I.), the chromatin being surrounded by cytoplasm and the spores thus formed partially or entirely separated from the parent organism.

After separation a resistant cyst wall, or sheath, as Schaudinn called it, is formed around each spore. This sheath at first has a double outline and is colorless, but soon becomes yellowish or brownish in color and the contents obscured. These little cysts measure from three to seven microns in diameter and do not stain well by any method which is used in the study of amebæ, so that it is

impossible to follow the development of the parasite within the cyst.

The morphology of *Entameba histolytica* in preparations stained with the Wright or Giemsa stain is very similar to that already described, with the exception that the ectoplasm and endoplasm are well differentiated, the ectoplasm staining a very dark blue while the endoplasm takes a light blue color. The chromatin of the nucleus stains a beautiful pink or red color, bacteria a very dark purplish red, and the erythrocytes a yellowish or greenish yellow. The stains must be well extracted in order to obtain fine morphological details, and the poor results obtained by some observers have been due to failure in this particular.

The nuclear membrane and karyosome are well differentiated in properly stained specimens, the chromatin staining pink or red, while in those organisms undergoing gemmation or sporulation the chromidia throughout the cytoplasm stain intensely and are beautifully shown. Owing to the intense action of the chromatin staining element in these stains the nucleus, as well as the chromidial rods, threads, and masses appear considerably larger than in specimens stained with hematoxylin, but careful extraction of the stain will remedy this defect, although it must be admitted that even in the best stained specimens the minute morphological details of the organism do not show as clearly as in specimens properly stained with hematoxylin after fixation with sublimate alcohol.

In specimens stained with the Wright stain the nucleus, after it has given up its chromatin to the cytoplasm, stains a duffuse grayish blue color, thus indicating that all of the chromatin has been distributed to the cytoplasm, the remains of the nucleus constituting a residual degenerative body.

Degeneration forms. — Degenerative forms of *Entameba histolytica* are frequently observed, the nucleus staining atypically or not at all, while the cytoplasm is poorly stained or filled with vacuoles. Very large parasites are seen entirely filled with erythrocytes, the nucleus being invisible or much swollen in appearance. Many of these degenerative

forms are impossible to classify and may be mistaken for *Entameba tetragena* or *Entameba coli*. Indeed, as Hartmann has shown, Schaudinn himself worked with both species without differentiating *Entameba tetragena* and undoubtedly confused degenerative forms of the latter organism with *Entameba histolytica* and vice versa. However, if one has studied the species in both living and stained preparations these degenerative forms will cause little confusion and the study of the life cycle will enable one to be sure of the identity of the parasite under observation. The distinct morphology of *Entameba histolytica*, its peculiar method of reproduction by sporulation, and the fact that no cysts are ever found in pure infections with this species at all comparable in appearance with those of *Entameba coli* or *Entameba tetragena*, definitely prove the right of *Entameba histolytica* to be considered a distinct species.

Fragmentation of the parasite into two or several fragments is often observed in living organisms, and sometimes fragmented organisms are seen in stained preparations. Some authorities have stated, without any evidence whatever, that such fragmented parasites have been mistaken by Schaudinn and others for sporulating parasites, but such a mistake would be impossible for one who has carefully studied this organism, as the fragments are irregular in size and shape and do not possess a nucleus as do the amebæ produced by sporulation.

The not infrequent occurrence of clumps of bacteria or protozoan parasites within amebæ should be noted, as such bodies might be mistaken for the chromidial threads or masses present in *Entameba histolytica* during sporulation. I do not believe that such inclusions would cause difficulty to the trained observer, but it is well to remember that they occur and must be differentiated from chromidia.

The study of the morphology and life cycle of *Entameba histolytica* in fresh preparations of feces containing the parasites should not be neglected, for as much can be learned from such preparations as from the best stained specimens and much of the confusion regarding this species has arisen

because investigators have relied entirely upon stained preparations.

ENTAMEBA TETRAGENA. (Viereck, 1907.)

Entameba tetragena is a pathogenic ameba which has been found in East Africa, Farther India, China, the Philippine Islands, South America, Panama, and the United States. In the past it has been frequently confused with *Entameba histolytica* and at the present time it is still uncertain which of these organisms is most prevalent in localities where amebic dysentery is common. From my own experience I would be inclined to believe that *Entameba histolytica* will be found to be the most common in the Philippine Islands, as this species was responsible for the majority of cases of dysentery returned from those islands between 1899 and 1905, but it will probably be found that in certain localities *Entameba tetragena* is the prevailing species, as would be indicated by Whitmore's²⁸ results in the examination of dysentery patients in Manila, already noted. In South America and in Africa, *Entameba tetragena* appears to be the common cause of amebic dysentery, although both species are found in these countries as well as in Panama and the United States.

The morphology of this species is well shown in wet-fixed, hematoxylin stained preparations and also in specimens stained with the Wright or Giemsa stain, if care is observed in extracting the stain by washing thoroughly in distilled water. The morphology varies in different stages of development and many preparations must be examined before the various stages in the life cycle can be demonstrated.

Vegetative stage of development. — In specimens wet-fixed and stained with hematoxylin the following morphological details may be observed in this organism:

The size of the parasite varies considerably. Hartmann gives from twenty-five to forty microns as the usual diameter, but I have seen organisms as small as fifteen microns in

diameter and as large as forty-five microns, and have found the average diameter to be about thirty-five microns.

In shape *Entameba tetragena* is roughly spherical or oval in stained preparations in most instances, but occasionally very irregular forms are observed where the organisms have been fixed while in motion.

There is no distinction between the ectoplasm and endoplasm unless the specimens have been fixed with osmic acid vapor, when the two portions of the cytoplasm are well differentiated. The ectoplasm is finely granular in appearance while the endoplasm is coarsely granular and contains bacteria, crystals, vacuoles, and, not infrequently, red blood corpuscles. The vacuoles generally vary in number from one to four or five, but dimly stained areas are almost always present, similar to those in *Entameba histolytica*, which are probably small digestive vacuoles; often the cytoplasm is stained very faintly or not at all.

The nucleus is well defined in both living and stained specimens, measures from five to eight microns in diameter, and is rich in chromatin. The nuclear membrane is of considerable thickness, as shown in hematoxylin stained specimens, and small dots of chromatin sometimes occur upon its inner surface. In the young amebæ most of the chromatin is collected in a mass at the center of the nucleus forming a deeply stained karyosome, but in the fully developed parasite the chromatin has a characteristic arrangement within the nucleus, either being scattered upon the inner side of the nuclear membrane and as minute deeply stained masses upon the linin network between the membrane and the karyosome, or as larger, irregularly shaped masses upon the inner side of the nuclear membrane. The karyosome is very characteristic in this species, being large, irregular or roughly spherical in shape, and sometimes appears to have a distinct membrane. It often appears connected with the nuclear membrane by delicate filaments, forming a dimly stained network (the linin network) upon which occur minute, deeply stained dots of chromatin. A centriole is generally present consisting of a deeply stained spherical dot or mass

of chromatin surrounded by a well marked unstained halo, which is not present in the nucleus of any other ameba I have studied. The appearance of the nucleus in stained preparations is well shown in the figures illustrating this species.

The only form of reproduction which has been observed during the vegetative stage is simple division. The centriole may be seen divided into two minute dots lying within the karyosome, while later the karyosome divides, followed by the division of the entire nucleus and the body of the ameba. The division of the nucleus occurs by a primitive mitosis, but much imagination is required to see in any of the nuclear changes during division the mitotic figures which are commonly observed in cells showing typical mitotic division.

Hartmann²⁹ has described certain cyclical changes occurring in the nucleus of *Entameba tetragena* which he considers characteristic, and I have been able to observe several of the changes in the appearance of the nucleus of which he writes. The karyosome sometimes appears as a fine network enclosing an unstained area within which lies the centriole, while the outer border of the karyosome is formed of deeply stained dots of chromatin arranged in a circular manner. Again, the karyosome may stain uniformly throughout with the exception of a clear unstained halo around the centriole, or the halo may be absent, the centriole appearing as a deeply stained, almost black mass within the karyosome. Rarely the entire karyosome is surrounded by an unstained area, the remainder of the nucleus presenting a dimly stained network having arranged upon it deeply stained dots of chromatin or the chromatin may be arranged in band-like masses near the nuclear membrane. These changes in the morphology of the nucleus are certainly very characteristic of this species for I have never observed anything approaching them in the nucleus of *Entameba coli* or *Entameba histolytica*.

Besides the typical vegetative forms which have been described, organisms are seen in which the cytoplasm contains chromidia evidently derived from the nucleus. These

chromidial masses vary much in size and shape but are usually spherical, oval, or spindle shaped, and of considerable size. The spindle shaped masses of chromatin are frequently observed and in the degenerative forms the chromatin may occur in the form of long delicate threads arranged in bunches throughout the cytoplasm. It has been shown that chromidial rods, threads, and small masses also occur in *Entameba histolytica*, but in *Entameba tetragena* the masses are generally much larger and are not arranged about the periphery of the organism as is the case in *Entameba histolytica* nor are they budded off from the parasite to form young amebæ or encysted spores. Nevertheless, this stage of the development of *Entameba tetragena* has caused the organism to be confused with *Entameba histolytica* in the past and the mistake may easily be made by one unaccustomed to the study of these parasites.

Encysted stage of development. — When conditions are unfavorable to vegetative existence *Entameba tetragena* forms cysts containing four daughter amebæ. These cysts are not observed in every infection and therefore it is not always possible to have the aid of these very characteristic bodies in the differentiation of this species.

The organisms which become encysted always contain more or less chromidia in the cytoplasm and when the cyst is fully developed this chromidia will be found collected into one or more spindle shaped masses, which stain deeply and are of large size. The cysts measure from seven to twenty microns in diameter, the average diameter being about fifteen microns. The small cysts are probably degenerative bodies as it is seldom that any division of the nucleus can be seen within them, and often the nucleus is absent. A double contoured membrane is often observed in fresh preparations, but is generally obscured in stained preparations. The cytoplasm, except for the masses of chromatin already mentioned, stains uniformly throughout, the organism before encystment having rid itself of bacteria, erythrocytes, and other foreign bodies. The nucleus in the young cysts is

round or oval in shape, and prior to division presents the same morphology as in the vegetative stage of development, although the nuclear membrane often appears thickened and the periphery of the karyosome stains very intensely. The primary division of the nucleus within the cyst, so far as shown in stained specimens, is very similar in morphology to that occurring during simple division, but a very long, atypical spindle is formed, the chromatin being scattered in deeply staining dots throughout it or collected in a crescent shaped body at each pole. Each of the two nuclei produced by the primary division possess a nuclear membrane and contain considerable chromatin collected in a mass near the center or at one pole, while chromatin granules are present arranged quite uniformly upon a delicate network which stains very dimly. These two nuclei each divide, thus producing a four-nucleated cyst, and this number of nuclei is never exceeded in this species, a fact which serves to distinguish these cysts from those of *Entameba coli*, which may contain more than the eight nuclei which are always found when the cysts of the latter parasite are fully developed. Thus, if only four nucleated cysts are present in an infection one may be sure that *Entameba tetragena* is the species present, while the eight-nucleated cyst is just as characteristic of *Entameba coli*.

In the cysts showing four nuclei the spindle shaped masses of deeply staining chromatin already mentioned are often observed to have disappeared, thus indicating that resorption of this substance has occurred. Some authorities have suggested that these masses are extruded from the organism, but as they are present after the cyst wall is fully developed I believe that it is very improbable that extrusion occurs.

The staining reactions of *Entameba tetragena* with the Wright and Giemsa stain are similar to those described, but here, as in the case of *Entameba coli* and *Entameba histolytica*, the chromatin staining is heavier and fine morphological details are hidden unless the stains are well extracted. With these stains the ectoplasm and endoplasm are not as well differentiated as in the other parasitic amebæ of man, the

cytoplasm generally staining a deep blue throughout. The nuclear chromatin and chromidial bodies stain a pink or red, while bacteria, erythrocytes, etc., stain as in *Entameba histolytica*. The nuclear membrane stains a dark blue unless very rich in chromatin.

Degenerative forms of this species are frequently observed in stained preparations and vary greatly in appearance. Organisms are seen entirely filled with vacuoles, the nucleus having disappeared. The nucleus may stain atypically, the karyosome filling nearly the entire nucleus, thus causing it to assume the appearance commonly observed in the nucleus of cultural amebæ; the centriole is often absent while the nuclear membrane may appear much thickened and very irregular in outline. In some instances the nucleus is broken up and the chromatin is scattered in the cytoplasm in the form of spherical bodies or threads, and such organisms are difficult to differentiate from the pre-cystic forms described.

Degenerated cysts are sometimes observed in which the nucleus has disappeared or an atypical nucleus is present surrounded by deeply stained masses of chromatin or by vacuoles. All of these degenerative forms are easily recognized when carefully studied and should lead to no confusion in the recognition of the species, although they have done so in the past.

AMEBA LOBOSPINOSA. (Nov. Sp.)

The cultural ameba herewith described and which I have named *Ameba lobospinosa* because of the peculiar morphology of its pseudopodia, has a most interesting history. It was first cultivated from a dysenteric stool by Doctor Musgrave, of Manila, who described it as *Ameba* 11524 of his series of cultural amebæ, and one of the cultures I have studied is descended directly from a culture he sent to the Army Medical Museum during 1905. I have also studied two other cultures of the same species originally sent to the Museum by the United States Army Board for the Study of

Tropical Diseases, from Manila, in May, 1908. It will thus be seen that this ameba has been cultivated for a period of nearly six years from the Musgrave culture and a period of three years from the Tropical Board cultures, and during this time the morphology of the organism has not changed and the life cycle remains the same as in the original cultures. One of the Tropical Board cultures was obtained from a dysenteric stool while the other was obtained from the pus of an amebic abscess of the liver.

In a future communication I hope to describe this ameba in detail, but here will only mention the chief morphological characteristics. The organism is described as a new species because of the characteristics of the pseudopodia which serve to distinguish it from any other cultural ameba I have studied.

In the living organism the ectoplasm is well differentiated from the endoplasm when the ameba is in motion but cannot be differentiated when motion is absent. The pseudopodia, composed of clear refractile ectoplasm, may be either lobose or typically spinose in shape, as first noted by Musgrave, and not infrequently spinose prolongations of a lobose pseudopodium may be observed. The spinose pseudopodia are extended very rapidly and often as rapidly withdrawn, so that sometimes it is rather difficult to distinguish them. The pseudopodia are very frequently multiple, as many as six or even eight being projected at the same time from the periphery of the ameba. Lobose pseudopodia are present when the organism is actively motile, although at the same time spinose pseudopodia may be observed. A well defined nucleus is present and a single contractile vacuole. In the vegetative stage this species reproduces by simple division, the nucleus showing well-defined mitotic figures during division. Under unfavorable cultural conditions this ameba encysts readily, each cyst containing only one nucleus, and, when conditions are again favorable, a single ameba is liberated from the cyst.

Vegetative stage of development. — In specimens fixed in

sublimate alcohol and stained with Delafield's hematoxylin this ameba resembles quite closely the one described by Whitmore³⁰ as *Ameba limax*, Subspecies M.I., which he obtained in cultures from dysenteric stools in Manila, but varies in size and in the peculiar character of the pseudopodia.

The size of this ameba varies between eight and twenty-five microns in diameter, the average being about sixteen microns. In shape the organisms are usually roughly spherical or oval but sometimes irregular forms are observed in those instances in which the amebæ have been fixed while in active motion.

The nucleus is very rich in chromatin which is collected in a compact, deeply stained, spherical mass, nearly filling the nucleus, and forming a very large karyosome. This mass, or karyosome, is surrounded by a clear unstained area devoid of chromatin, which, in turn, is surrounded by a zone of fine chromatic granules forming an irregular and poorly stained nuclear membrane. A centriole is not generally visible in the karyosome, owing to the dense staining of the latter, but in specimens which have been thoroughly extracted with acid alcohol a centriole is sometimes observed, consisting of one or two deeply stained dots lying near the center of the karyosome. In this species there is no evidence of the achromatic network which is so well seen in the nucleus of *Entameba tetragena*, nor are free chromatin granules observed in the unstained area surrounding the karyosome or upon the inner side of the nuclear membrane. In many organisms the nuclear membrane cannot be differentiated from the cytoplasm, the nucleus consisting of a large karyosome surrounded by an unstained border.

The cytoplasm contains large numbers of the bacteria growing in symbiosis with the ameba, and in many organisms a single large vacuole may be observed while small unstained oval or round areas are often noted, as in the parasitic amebæ.

Reproduction during the vegetative stage is often well

shown in stained preparations and consists in simple division, the nucleus showing well-defined mitotic figures. Organisms are observed in which the karyosome is greatly lengthened, as well as the entire nucleus, and sometimes evidences of the division of the centriole are noted in well extracted specimens. A later stage in division is shown in organisms having deeply stained crescentic bodies at each pole of the nucleus connected by fine filaments of poorly stained material, forming a well defined spindle. An equatorial plate is not infrequently observed consisting of a band of deeply stained chromatin stretching across the spindle midway between the polar bodies, and often a distinct division of this plate into two rows of deeply staining granules may be observed. Still later the equatorial plate and spindle are observed to have disappeared and the nucleus is divided into two portions, spherical in shape, and having a compact karyosome and a diffusely stained nuclear membrane.

In this species the mitotic division of the nucleus may be easily studied and is very typical when compared with the very primitive form of mitosis observed in the nucleus of the parasitic amebæ.

I have seen no evidence of budding in this species, although not infrequently small portions of cytoplasm are pinched off from the organism. These are not nucleated and undergo no further development, and therefore the process is probably a degenerative one.

Cystic stage of development.—*Ameba lobospina* produces cysts readily whenever the cultural conditions are unfavorable to vegetative reproduction. The cysts measure from eight to fifteen microns in diameter, the average being about ten microns. The young cysts are spherical in shape, but as they grow older the cyst wall thickens and contracts unequally, thus producing irregular forms, often quadrilateral or pyramidal in shape. The cyst wall is smooth and has a double contour at first but later may become rough or even appear mammillated. Old cysts often have a very thick wall and appear cracked or folded over in places as shown

in Plate II. (IV.). The cytoplasm of the cyst is generally free from vacuoles and bacteria and contains a single nucleus having a large karyosome surrounded by an unstained area. The nucleus is usually situated near the center of the cyst. I have never seen more than one nucleus in a cyst nor have I observed any evidences of division of the nucleus within the cyst. Under favorable conditions the cyst liberates a single ameba and it therefore follows that in this species encystment is a protective process only, and not also reproductive as in the parasitic amebæ of man.

In this species of ameba I have never observed any evidence of spore formation which has been described for some cultural amebæ by Walker,³¹ Greig and Wells,³² Noc,³³ and others, but upon one culture plate small spherical protozoan organisms developed which, when engulfed by the amebæ, resembled the spores described by some writers, especially in the stained preparations. I have seen similar inclusions in the parasitic amebæ and for this reason care should be taken to differentiate the bodies before an ameba is reported as producing spores.

Pathogenicity.—This species of ameba was described by Musgrave³⁴ as capable of producing dysentery in monkeys, and, in a single instance, in man. Its isolation from a dysenteric stool was significant and Musgrave's experiments upon monkeys were almost conclusive of the pathogenic nature of this organism. However, later researches have shown that the amebæ which have been cultivated from dysenteric stools and the pus of liver abscesses are really free-living species, non-pathogenic in nature, and their presence in the stools and pus can be easily explained by contamination of the material or the culture media by dust containing the cysts of the amebæ or their spores. In the stools, also, the presence of free-living species may be explained by the ingestion of food or drink containing the cysts or vegetative forms. Recently Liston³⁵ has recorded the cultivation of an ameba upon plates exposed to the air in an Indian jail which is identical with an ameba he had

previously cultivated from liver abscess pus, thus demonstrating how easily such material as feces and pus may become infected with free-living amebæ.

The results of the experiments upon monkeys are vitiated by the liability of these animals to naturally acquired amebic infection and also by the possibility of the contamination of the material used in the experiments by the spores of *Entameba histolytica* or the cysts of *Entameba tetragena* in a locality like Manila, where amebic dysentery is endemic. The same criticism applies to the single experiment upon man, as described by Musgrave.

One cannot, therefore, accept the pathogenicity of this species as proven, and as its morphology and life history agree with the free-living amebæ and differ entirely from the parasitic amebæ of man, we are justified in regarding it as a free-living species having nothing whatever to do with the etiology of amebic dysentery.

CONCLUSIONS.

The morphological studies given in this paper might be described in much greater detail, but I believe that they are sufficient to prove that the organisms under consideration differ greatly in morphology and in their life history, and that each possesses characteristics which entitle it to specific rank.

As regards the differentiation of *Entameba coli*, *Entameba histolytica*, and *Entameba tetragena* it may be stated that these species may be recognized without much difficulty by one trained in the study of this class of organisms, and while it may not be very important, from a practical standpoint, to differentiate the pathogenic species from one another, it is most important that the harmless *Entameba coli* be differentiated from the pathogenic forms, as otherwise many individuals will be treated for amebic dysentery when in reality only the harmless *Entameba coli* is present. Success in the recognition of the various species of amebæ can only come

from practice, and it is most unfortunate that the examination of the feces is so much neglected by the student and practitioner. When one remembers that *Entameba coli* may be found in from five to fifty per cent of healthy individuals in almost every locality it is evident that one may secure material for the study of this parasite without difficulty and thus become able to differentiate it from the pathogenic species concerned in the production of dysentery. In many regions where amebic dysentery is endemic, material is available for the study of all of the parasitic amebæ of man, and a diagnosis of amebic dysentery should not be made until one is sure that a pathogenic species is present.

The parasitic amebæ of man are entirely distinct in morphology and life cycle from the cultural species described in this paper, and it is difficult to understand how the latter species could be confused with any of the amebæ which are true parasites of man. The morphology of the nucleus of *Ameba lobospinosa*; the presence of both lobose and spinose pseudopodia; the contractile vacuole; and the fact that the cysts contain but one nucleus, should serve to distinguish this species from any of the parasitic amebæ so far described as occurring in man.

Williams,³⁶ in a recent paper describing the cultivation of certain amebæ in pure culture, describes this species under the name *Ameba coli* and considers it a parasitic species, apparently because it was isolated from a dysenteric stool. However, as it is really a free-living species, the name *Ameba coli* cannot be applied to it, for it differs markedly both in morphology and life cycle from *Entameba coli* (*Ameba coli*, Loesch), and the conclusions of Williams are erroneous because she had no opportunity of comparing this species with the parasitic amebæ of man. The work of such zoölogists as Hartmann, Prowazek, Schaudinn, Doflein, and others, has proven beyond question the value of the morphology of amebæ and of a study of their life cycle in the differentiation of the free-living and parasitic species, and conclusions based entirely upon the study of cultural

species are practically valueless unless such species are actually compared with the true parasitic amebæ of man. I have studied this together with the three parasitic species described in this paper and am able to state that it is entirely distinct from them and is a typical free-living ameba.

Note.—In the October, 1911, number of the Philippine Journal of Science, Section B, which reached me recently, there occurs a very important article by E. L. Walker upon the "Amebæ in the Manila Water Supply, in the Intestinal Tract of Healthy Persons, and in Amebic Dysentery," in which the author confirms the occurrence of *Entameba coli* and *Entameba histolytica* and concludes that all the amebæ which can be cultivated from the intestinal tract of man or animals are free-living species and differ entirely in their morphology and life cycle from the parasitic species. His principal conclusions are:

"The ameboid organisms cultivable from the intestinal tract of man, both from healthy persons and from cases of amebic dysentery, also belong to the genus *Ameba* Ehrenberg.

"The cultivable species of the genus *Amebæ* are not parasitic in the intestinal tract of man; when obtained in cultures from the intestine they probably are derived from cysts of amebæ that have been ingested with water or food and have passed unchanged through the intestinal tract.

"The ameboid organisms parasitic in the intestinal tract of man belong to a distinct genus, *Entameba*, *Casagrandi* and *Barbagallo*.

"The entamebæ are strict or obligatory parasites and are incapable of multiplication outside of the body of their host. They cannot be cultivated on Musgrave and Clegg's medium."

The author also concludes that *Entameba coli*, the non-pathogenic species, which includes *Entameba nipponica*, and a presumably pathogenic species, *Entameba histolytica*, which includes *Entameba tetragena*, should be recognized and that it is possible to differentiate these parasites with the microscope.

This paper is of special interest because some of the cultures with which Williams worked had been isolated from the intestine of man and animals by Walker, in 1908, and were considered as parasitic by him because they were cultivated from the intestine, and have also been considered as parasitic by Williams. It is now evident that these species can no longer be classed as parasitic, and therefore the conclusions drawn by Williams regarding their morphology and life cycle, so far as they relate to the parasitic species, are erroneous. The later researches of Walker confirm those of Schaudinn and other students of this subject as regards the nonidentity of these cultural species with the true parasitic species found in man and other animals. I cannot agree with him that *Entameba histolytica* and *Entameba tetragena* are identical, however, because I have seen many infections with the former in which four-nucleated cysts could not be demonstrated at any stage of the infection. It is interesting

to know that Walker found amebæ showing the nuclear characteristics of *Entameba histolytica* more frequently in infections in Manila than those showing the nuclear structure of *Entameba tetragena*, as that has been my own experience. I believe, at the present time, that these two species are distinct, but further research should definitely settle this important and interesting point.

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EXPLANATION OF THE PLATES.

(All figures drawn by the author.)

PLATE I.

I. UPPER GROUP. — *Entameba coli* stained with Giemsa stain.

A, B, and C. Vegetative organisms showing nuclear membrane, karyosome, and collections of chromatin upon the nuclear membrane and within the hyaloplasm. Vacuoles are also present.

D. An organism containing a protozoan parasite which might be mistaken for spores.

H. Division of the nucleus (primitive mitosis).

E. Partially divided ameba containing two nuclei.

F, G. Ameba resulting from simple division.

M. Schizogony of *Entameba coli*. Eight daughter nuclei in vegetative form.

N. Ameba resulting from schizogony.

I. Earliest stage in cyst formation. Cytoplasm clear of foreign bodies and nucleus showing collection of chromidial masses upon the inner side of the nuclear membrane.

K, L, O, P. Two and four-nucleated stage of reproduction within the cyst.

Q. Encysted form containing two large nuclei and a mass of chromatin.

R. Fully developed cyst of *Entameba coli* containing eight nuclei.

LOWER GROUP. — *Entameba coli*, fixed in sublimate alcohol and stained with Delafield's hematoxylin. Note the more delicate staining of the nucleus and the greater detail obtained with this method of staining.

A, B, C. Vegetative amebæ showing variations in the structure of the nucleus.

D. An organism during schizogony, containing eight nuclei.

E. Mitotic division of the nucleus as observed in this species.

F. A fully developed cyst of *Entameba coli* containing eight daughter nuclei.

G. The four-nucleated cystic stage of *Entameba coli* sometimes mistaken for the cyst of *Entameba tetragena*.

H. Two-nucleated cyst of *Entameba coli*.

I. Young amebæ originating from the cysts of *Entameba coli*.

K. Fully developed cyst in which the cystic membrane is apparently absent.

L. Degenerated cyst of *Entameba coli*, filled with vacuoles, and containing masses of chromatin. No nucleus is visible.

II. *Entameba histolytica* stained with Giemsa stain.

A. Organism showing distinction between the ectoplasm and endoplasm, nucleus, and vacuole.

B. Organism showing vacuole and red blood corpuscle and nucleus containing minute karyosome and chromatin dots in the hyaloplasm.

C. Organism showing nucleus and numerous red blood corpuscles.

D. Organism in first stage of nuclear division, showing division of the karyosome and minute dots of chromatin in the hyaloplasm.

E. Organism showing later stage of nuclear division, the polar bodies being connected by a filament of chromatic substance.

F. First stage of formation of spore cysts; the nucleus distributing chromatin to the cytoplasm.

G to I. Stages in the process of formation of spore cysts, the chromatin being distributed to the cytoplasm and collected in threads or masses, while the nucleus is observed as a flattened body crowded against the periphery of the parasite.

L. Degenerated parasite containing vacuoles and free chromatin.

K, M, N. *Entameba histolytica* in the final stage of the formation of spore cysts. The free chromatin has collected at the periphery, and surrounded by a small amount of cytoplasm, is being budded off from the parent organism.

O. Degenerated organism filled with vacuoles and free from chromatin. The nucleus stains abnormally and there is no distinction between the ectoplasm and endoplasm.

P. *Entameba histolytica* filled with erythrocytes, the nucleus being crowded to the periphery and staining abnormally.

PLATE II.

III. UPPER GROUP. — *Entameba tetragena* fixed in sublimate alcohol and stained with Delafield's hematoxylin. Note the greater delicacy of the staining when compared with the staining with the Giemsa method.

A. A vegetative parasite showing three erythrocytes in the cytoplasm and a nucleus in which the nuclear membrane, and the karyosome with its centriole are shown.

B. A vegetative organism showing thick nuclear membrane and karyosome containing a centriole.

C. A vegetative parasite containing vacuoles and nucleus showing karyosome containing a centriole surrounded by an unstained area.

D. A degenerative form filled with vacuoles and showing abnormal appearance of the nucleus.

E. Precystic form of *Entameba tetragena*.

G. Another precystic form which is more typical in that the free chromatin in the cytoplasm is visible. The form E would probably degenerate before the cyst wall was fully formed.

F. A cystic form of *Entameba tetragena* showing two chromatin spindles in the cytoplasm and a nucleus having a centriole surrounded by an unstained area and a definite network upon which are arranged dots of chromatin.

H. An encysted form showing a very large mass of chromatin and a nucleus containing a karyosome and-centriole.

I. Two-nucleated cyst of *Entameba tetragena* showing mass of free chromatin and the morphology of the nuclei after division.

K. Fully developed cyst of *Entameba tetragena* containing four daughter nuclei and a mass of chromatin.

L. Degenerative form of *Entameba tetragena* containing some free chromatin and a nucleus in which the karyosome stains deeply and nearly fills the nucleus. This form might easily be mistaken for a free living ameba.

M. Illustrating the typical nuclear structure of *Entameba tetragena*. Note the large karyosome containing a centriole surrounded by an unstained area.

LOWER GROUP. — *Entameba histolytica* fixed in sublimate alcohol and stained with Delafield's hematoxylin.

A and B. Vegetative organisms showing vacuoles and typical morphology of the nucleus. No distinction between the endoplasm and ectoplasm.

C. Vegetative form of *Entameba histolytica* showing the type of mitosis during simple division.

D. First step in the formation of spore cysts. The distribution of the chromatin by the nucleus to the cytoplasm.

E, F, and H. Organisms showing chromidia in the cytoplasm arranged in rods, threads, and masses, the nucleus being flattened out against the periphery and staining poorly.

G. A degenerative form of *Entameba histolytica* filled with vacuoles and with an atypical nucleus.

I and K. Budding of the spore cysts from the periphery of *Entameba histolytica*.

L. Illustrating the typical nuclear structure of *Entameba histolytica*.

IV. UPPER GROUP. — *Entameba tetragena* stained with Giemsa stain.

A, B, C. Vegetative organisms. Note that the nuclear membrane and karyosome stain very heavily and are not as well differentiated as in specimens stained with hematoxylin.

D. Precystic form containing masses of chromatin in the cytoplasm.

E. Degenerative form containing vacuoles, masses of chromatin, and an atypically stained nucleus.

F. Two-nucleated stage of the cyst of *Entameba tetragena*, showing heavy staining of the nuclear membrane and karyosome. Two masses of chromatin are present.

G. Fully developed cyst of *Entameba tetragena* containing four nuclei and one mass of chromatin.

H. Illustrating the type of nucleus as observed in *Entameba tetragena* in specimens stained with the Giemsa stain.

LOWER GROUP. — *Ameba lobospinosa* stained with Delafield's hematoxylin after fixation with sublimate alcohol.

1, 2, and 3. Vegetative organisms showing the large contractile vacuole and the typical nucleus containing a deeply stained karyosome almost filling the nucleus.

4. A vegetative ameba in which the nucleus has divided.

5, 6. Vegetative amebæ in which the nucleus is dividing. Polar bodies are present connected by filaments and a well marked equatorial plate is apparent.

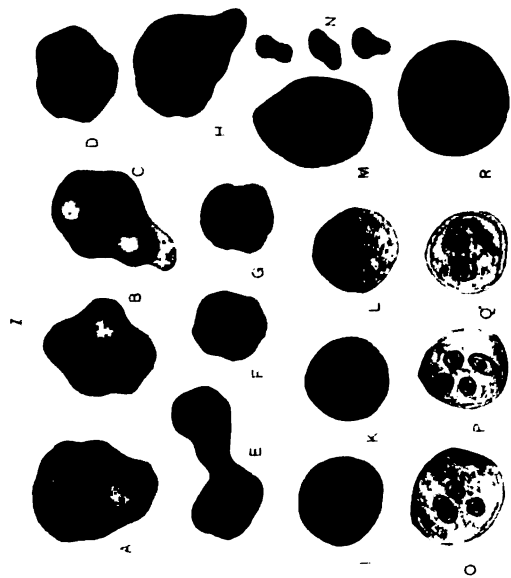
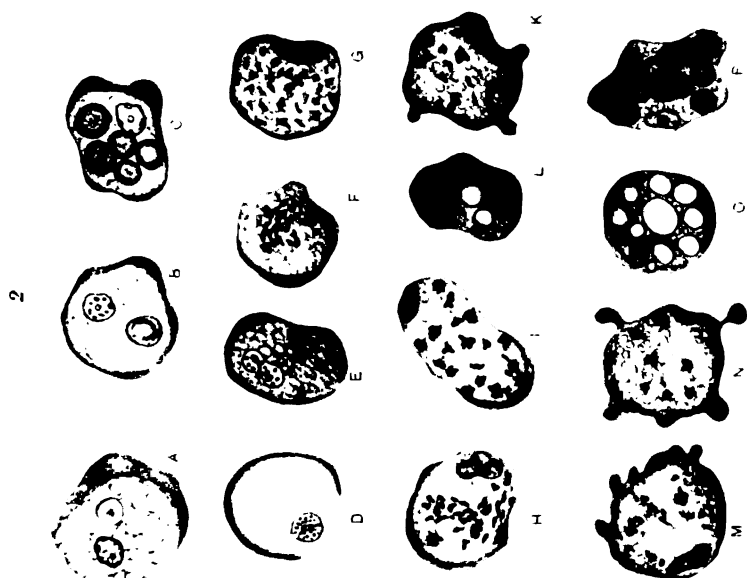
7. Degenerated vegetative ameba filled with vacuoles and with atypically staining nucleus.

8. *Ameba lobospinosa* containing a protozoan organism. These forms have been mistaken for sporulating amebæ.

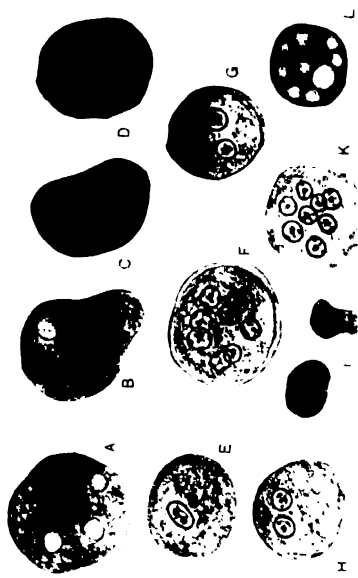
9 and 10. Encysted forms of *Ameba lobospinosa* during the first few days in cultures.

11 to 18 (except 14). Various cystic forms of *Ameba lobospinosa* showing the character of the cyst wall in the older cysts. At 12 the cyst contains two vacuoles and the cyst membrane is folded in, an appearance frequently observed in cultures which have become dry; 15 and 17 represent cysts in which the cyst wall is cracked and a nucleus cannot be distinguished; 16 represents a cyst filled with deeply staining granules of chromatin derived from the degenerated nucleus; 18 is a cyst in which only the cystic membrane is visible, the ameba having escaped from the cyst.

14. A fragmenting ameba frequently mistaken for a budding organism before the separation of the fragments.



UPPER GROUP



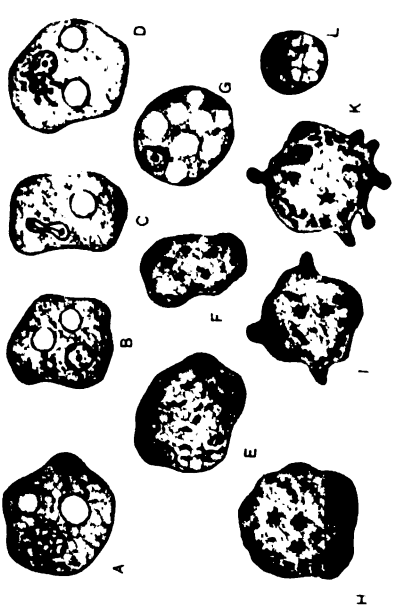
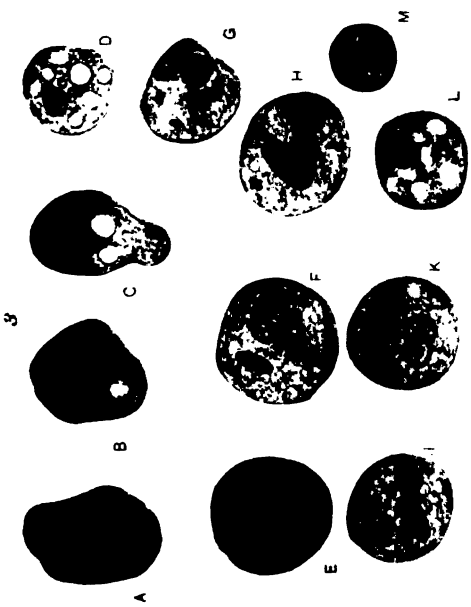
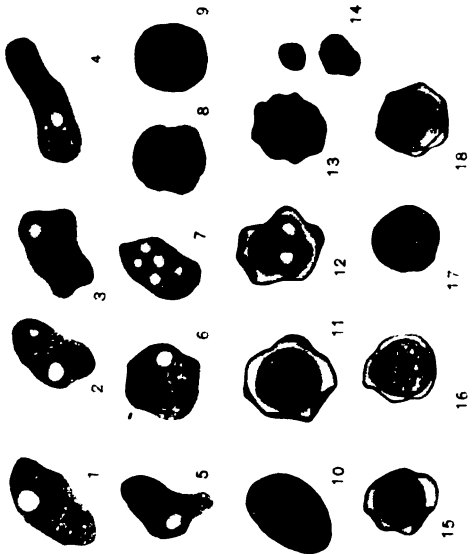
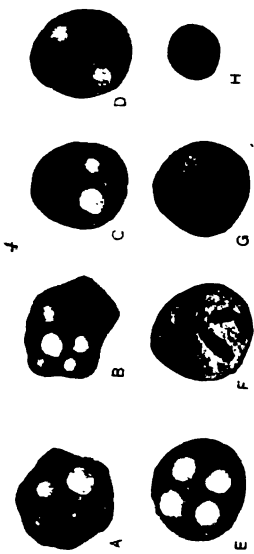
LOWER GROUP

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UPPER GROUP

LOWER GROUP



UPPER GROUP

LOWER GROUP

THE MOLASSES PLATE METHOD: A MODIFICATION OF THE
HUBER-SCHMORL-OBREGIA METHOD.*

ALDRED SCOTT WARTHIN, PH.D., M.D.

*(Professor of Pathology and Director of the Pathological Laboratories in the
University of Michigan, Ann Arbor, Michigan.)*

(From the Pathological Laboratories of the University of Michigan, Ann Arbor.)

Numerous methods have been devised for the purpose of staining many paraffin or celloidin sections at one time, either by attaching them to a slide or glass plate, or by manipulating the sections in single large films or sheets after they have been cut singly or serially. These methods have been applied particularly to the preparation of sections for teaching or research purposes when it is desired that a large number of sections of one preparation should be stained evenly, or when they are to be studied in series. They are also of great convenience in practical diagnostic work by facilitating the staining of a large number of sections at one time. Those methods by which the sections are manipulated as one large section after being transferred into a sheet or film have proved to be the most convenient and satisfactory. Of these Giacomini's collodium-gelatin (1885), Weigert's paper-collodium (1885), Strasser's paper-gum collodium (1895), and the Obregia dextrin-sugar (1890) methods have been most generally used; the latter, particularly in teaching laboratories, has been found to be a most valuable method for many purposes.

Obregia used a solution of three parts cane-sugar syrup, two parts ninety-five per cent alcohol and one part of transparent dextrin; this is spread over the slide or glass plate, which is then allowed to dry in the drying-oven. On this dry plate celloidin or paraffin sections were transferred from a sheet of tissue — or closet — paper moistened with ninety-five per cent alcohol, on which sheet the sections are previously arranged with their satiny sides upwards. The paper holding the sections is turned over upon the sugar-dextrin plate and gently pressed down; the paper is then

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carefully lifted off leaving the sections adherent to the glass plate. After carefully blotting the sections with a pad of absorbent paper a three per cent solution of photoxylin is poured over the plate; it is allowed to dry for a few minutes, and then immersed in water, where the sugar-dextrin dissolves and the photoxylin sheet floats off to be treated as one section.

Gulland (1893) modified this method by using a solution of three hundred cubic centimeters of a syrupy solution of cane-sugar in distilled water, twenty cubic centimeters of absolute alcohol and ten cubic centimeters of a syrupy solution of dextrin in distilled water.

Schmorl (1897) advised a modification of the Obregia-Gulland method that proved so superior to all others advised for the same purpose that it became generally adopted, particularly for paraffin work. Schmorl employed two solutions:

Solution I.: Cane-sugar solution (1 : 1), 300 cc.
 80 per cent alcohol, 200 cc.
 Yellow dextrin solution (1 : 1), 100 cc.

This solution is poured over a perfectly clean glass plate, the excess drained off, and the plate warmed over the flame. The paraffin sections are then arranged upon the moist warm sugar-dextrin solution with their satiny side downwards, the plate being warmed sufficiently to spread the sections out perfectly flat without melting the paraffin. After the plate has been covered with sections it is put into the drying oven until dry (three to twelve hours). When dry the plate is put into a dish of xylol to remove the paraffin, then into absolute alcohol to remove the xylol. From the alcohol the plate is taken up and covered over with a thin layer of Solution II.

Solution II: Photoxylin or celloidin, 10 cc.
 Absolute alcohol, 100 cc.
 Ether (pure), 100 cc.

The celloidin sheet is allowed to dry in the air for one to

two minutes, when the plate is put into warm water in which the sugar-dextrin dissolves and the celloidin film containing the sections floats up from the plate. The film is then transferred upon the glass plate into the desired solutions or stains. When ready for mounting the individual sections can be cut and mounted separately.

While this method was a great improvement over all preceding ones in that it permitted the imbedding of material in paraffin and its staining in celloidin sections; and was especially adapted to the preparation of serial sections, yet it had certain disadvantages. The pouring of the sugar-dextrin solution over the glass plates was a sticky and unpleasant procedure, it was a relatively expensive method, and the sugar-dextrin solution would often not dry quickly, and bubbles and crystals often formed in the plates during drying, thus spoiling the sections. Moreover, if the paraffin sections become folded as they are dropped upon the sugar-dextrin plate it is very difficult to flatten them out.

Huber and Snow (1906) improved the method greatly by floating paraffin sections directly on to a warm three to five per cent solution of Schmorl's Solution I. and then plating the sections from this. This method is much cleaner, less expensive and requires less time for drying in the incubator. There is also much less formation of bubbles and crystals; and the difficulty of obtaining flat sections is wholly obviated.

As in a large teaching and diagnostic laboratory where many sections must be handled daily, any modification of technical methods that will save time and cost without impairing results is most desirable, certain changes in the Huber-Schmorl-Obregia method were sought in my laboratory for several reasons. The large amount of the alcoholic sugar-dextrin solution used and its relatively high cost constituted an item of expense of sufficient importance to induce us to look for a cheaper substitute. Further, the time necessary for drying the sugar-dextrin plates, even of the water solution, made us desirous of using a solution that would dry quickly, be free from bubbles and crystals, and

yet possess a consistency differing from that of the sugar-dextrin solution. Accordingly many solutions of syrups were tried out, and it was found that practically all of them could be used as substitutes for the sugar-dextrin solution. The proprietary "corn-syrups" so-called were found to be well adapted for use in this method; but the cheapest and best substance was found to be the cheapest form of New Orleans molasses, the mixture known in the trade as "black" or "baking" molasses. The New Orleans variety is better than the black syrups called "Jamaica" or "West Indian." It retails ordinarily at twenty cents per gallon. Diluting the commercial molasses to a ten per cent solution, a gallon of the dilute solution costs but two cents, and this can be preserved indefinitely by a crystal of thymol or phenol added to prevent fermentation. So far as cost is concerned, therefore, nothing could be cheaper. Moreover, the molasses solution has a different consistency — more oily — than the sugar-dextrin. Sections are more easily slipped on to the glass plates, and when arranged do not come off or slide about so easily. The solution never forms bubbles or crystals during the drying process.

An agate pan filled with the dilute molasses solution is placed on a tripod near the microtome. It is kept warm by means of a low-flame Bunsen burner placed beneath it. As the paraffin sections are cut they are transferred directly from the knife by means of a camel's hair brush, and floated upon the warm molasses solution with their satiny sides downwards. The solution should be just warm enough to flatten the sections perfectly without melting the paraffin. When a sufficient number of sections to cover a plate have been obtained, the latter, having first been perfectly cleaned, is slipped into the solution at one end of the tray, and gradually immersed so that the floating sections can be pushed up or drawn up on it as far as desired. The sections are manipulated by means of a camel's hair brush. As the sections are arranged in rows the plate is lifted gradually out of the solution, until it has been completely

covered with sections. It is then lifted out of the tray and allowed to drain.

The section-covered plate may be dried in the drying-oven, over a Bunsen burner, or simply in the air, taking a few minutes or several hours according to the needs of the case. It is not necessary, however, to take the time for drying required by the sugar-dextrin solution. As soon as the plates are drained they may be quickly dried over a Bunsen burner and then flooded with absolute alcohol; after several minutes the plate is taken out of the alcohol and Schmorl's Solution II. is poured over the sections. This celloidin is allowed to set for a minute or two; and the plate is then immersed in warm water to detach the celloidin film containing the paraffin sections. This film is then handled by catching it by the two corners of one end with the fingers or better by a pair of forceps held in each hand. Handling the films with the bare hands should be avoided as desquamated epithelium becomes firmly attached to the celloidin and will appear as an artefact in the mounted section. Transferring the films on the glass plates from one solution to another is a waste of time. The films can be caught at two corners with the forceps, straightened with a quick jerking flap, and placed perfectly flat. They should not be allowed to float on the surface, nor should the solution containing them be exposed to dust, as the latter will adhere firmly to the celloidin. The trays should be covered if films are allowed to stand in them some time.

The film is transferred to a dish of xylol to remove the paraffin, then into ninety-five per cent alcohol, then into water and finally into the staining solution. After staining the film may be washed, dehydrated and cleared as desired. In the clearing solution the film may be cut into strips or single sections by means of a paper-cutter's wheel or by scissors. The use of the wheel-cutter is strongly recommended, as by it the film can be much more quickly and easily cut. The strips and sections are then mounted as desired.

This method of transferring paraffin sections into a celloidin sheet without first drying the plate and removing the paraffin saves a great deal of time when quick methods are desired. If the saving of time is not necessary the molasses plate may first be dried, then immersed in xylol to remove the paraffin, then placed in absolute alcohol, and the celloidin solution poured over it as soon as it is lifted out of the alcohol. The celloidin is allowed to set for a few minutes and the film is then detached in warm water, and carried through the same processes as above.

This method is now used in my laboratory to the exclusion of others for all ordinary diagnostic work and for the preparation of teaching material except in the case of certain specific staining methods that must be carried out on cover-glasses. It permits the handling of a large number of sections at a minimum of time and trouble. Believing firmly as I do in the value of giving students preparations to have as their own and to take away with them from the laboratory, rather than in the demonstration of stock sets of slides belonging to the laboratory, the molasses plate method becomes the most valuable practical method known to me for the rapid preparation of many sections at one time. When it is desired that students should do their own staining, paraffin sections may be cut and arranged in celloidin films which may then be cut into strips and given out in water or alcohol to the students for staining. The celloidin sheets may be rolled on test-tubes or glass rods, and preserved in eighty per cent alcohol for future staining, as in the case of single celloidin sections, but personally I have never found it of any advantage to do this, since new sheets can be so quickly made from the paraffin blocks which are more easily kept indefinitely.

The molasses solution may be used also for the rapid staining of paraffin sections on the slide, and for the staining of fresh or fixed tissues cut on the freezing microtome without imbedding. Serial sections of celloidin blocks may also

be arranged upon slides or plates covered with a dilute molasses; the slide or plate is then flooded with absolute alcohol and then drained; a thin celloidin is poured over it, and the plate immersed in warm water when the film containing the celloidin sections is liberated, and is then stained as desired.

Sections may be numbered on the molasses plate by marking the latter with a blue wax pencil after the paraffin sections have been floated on to it. The pencil markings will be transferred to the celloidin sheet, which will retain them through all solutions.

The success of the plate method will depend largely upon the condition of the glass plates when put into the molasses or sugar-dextrin solution. They must be clean or the celloidin film will not separate well. The plates may be kept in alcohol or acetic-acid-alcohol cleaning fluid until needed. The celloidin must also be of proper consistency, the film should be thin and cover the plate uniformly. It must not be allowed to set too much in the air before its detachment in water, or it will become tough and shrink. Discarded photograph plates when cleaned are useful for this method; and agate, glass, porcelain or rubber trays corresponding to them in size can be obtained in which to carry the plates through the various solutions. For the beginner in this method it is recommended that the slower method of drying the plate and removing the paraffin in xylol should first be tried, as some difficulty might be experienced by a beginner in judging just the right time for flooding the rapidly dried plate with absolute alcohol without floating off the paraffin sections.

To recapitulate, the molasses plate method offers the cheapest, quickest, and best method for the staining of large numbers of sections at one time.

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A COLORIMETRIC TEST FOR CHOLESTEROL.*

PAUL G. WESTON, M.D.

(*From the Pathological Laboratory, State Hospital for the Insane, Warren, Pa.*)

The observations herein described were made with a view to finding a method for making quantitative determinations of quantities of cholesterol too small to be conveniently handled and weighed.

The method which has been developed is based in principle upon Salkowski's test, the directions for which are given as follows: "A few crystals of cholesterin are dissolved in a small quantity of chloroform and treated with an equal volume of concentrated sulphuric acid. The solution of cholesterin first assumes a blood red color, and then gradually turns to a violet red, while the sulphuric acid appears dark red and shows a green fluorescence."¹ This describes the reaction fairly well when the amount of cholesterol present is not less than three milligrams to one cubic centimeter of chloroform. It should be stated, however, that the mixture first assumes a yellow color which rapidly becomes a deep red and then gradually turns to violet. This immediate reaction of which no mention is made in the usual text-book descriptions is of little significance when the cholesterol solution is comparatively strong; but a very weak solution reacts much more slowly, and each stage of the reaction calls for special attention.

For quantities smaller than two milligrams in one cubic centimeter of chloroform, the reaction may be described as follows: first both the solution and the sulphuric acid assume a greenish yellow color, which rapidly becomes deeper and less greenish; after a few minutes the solution appears amber colored, and no marked change will be noticed during the next hour. In the course of the succeeding six hours the solution gradually becomes pink in color, and finally violet.

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It is possible, however, to obtain the distinctive reaction in a much shorter time. After the mixture has stood for from fifteen to thirty minutes, the addition of a small quantity of chloroform causes the amber color to disappear, and in a few minutes the now colorless solution begins to show the pink tint which is characteristic of cholesterol. Later this pink gradually turns to a pale violet; this change of color may be hastened by decanting the solution from the sulphuric acid.

In order to standardize the test it was necessary to determine:

1. The proportions of chloroform and sulphuric acid most favorable for bringing out the reaction.
2. The time at which the characteristic color becomes relatively stable.
3. The most favorable method of handling the mixture.

One cubic centimeter of chloroform was chosen arbitrarily as a convenient quantity to be used in the experiments. The test-tubes used were one by ten centimeters. Preliminary tests showed that .0003 gram of pure cholesterol dissolved in one cubic centimeter of chloroform was sufficient to give an easily recognizable reaction, and a solution of this strength was used extensively in studying the effects of different variables.

To determine the amount of chloroform to be added for the purpose of bringing out the pink color, five test-tubes were set up, each containing .0003 gram of cholesterol in one cubic centimeter of chloroform. These were treated with equal amounts of sulphuric acid, and thirty minutes later received increasing doses of chloroform. The reactions are shown in Table I.

TABLE I.

Cubic Centimeters of Chloroform.	Reaction.
0.2.....	Muddy, yellowish pink.
0.4.....	Pink, slightly yellowish.
0.6.....	Pink, very slightly yellowish.
0.8.....	Pink.
1.0.....	Pink, approaching violet.

The effect produced by the addition of one cubic centimeter was more distinctive than that of the smaller doses, and this amount was decided upon as the most convenient dose.

The necessary amount of sulphuric acid was determined as follows: ten test-tubes were set up, each containing .0003 gram of cholesterol in one cubic centimeter of chloroform, and to these were added varying amounts of sulphuric acid, ranging from .1 to one cubic centimeter. The depth of the yellow color, both in the solution and in the sulphuric acid, varied inversely as the amount of acid used. When more chloroform was added, however, the ten tubes showed the same depth of pink. As .1 cubic centimeter of sulphuric acid appeared to give as good results as the larger amounts, this was adopted as the standard dose.

A special series of tests was made for the purpose of determining the most favorable time for the subsequent addition of chloroform. Six sets of test-tubes were prepared, three in a set, each set including solutions of .0002, .0003, and .0004 gram of cholesterol in one cubic centimeter of chloroform. All were treated with .1 cubic centimeter of sulphuric acid. At intervals of fifteen minutes, beginning at fifteen and ending at ninety minutes, one cubic centimeter of chloroform was added to each of the three tubes of a single set. The tubes receiving the chloroform fifteen minutes after the sulphuric acid presented a salmon pink, while those

receiving the chloroform later were less yellowish and more violet in hue. The six sets showed a gradation from salmon pink to violet pink. The fifteen-minute interval gave fairly satisfactory results, but the color was found to be more stabile if an interval of thirty minutes elapsed before the chloroform was added.

Variations in the quality of the pink tint have occurred by reason of a lack of uniformity in the handling of the test-tubes. Tests made to determine the effect of shaking indicated that, other things being equal, the more thoroughly the tube is shaken the more yellowish the solution and the longer the time required for bringing out the violet pink color. The extent of the contact between the solution and the sulphuric acid appears to be a more important factor than the amount of acid used.

As a result of much experimentation the following technic has been found to give very satisfactory results:

One cubic centimeter of a chloroform solution of cholesterol is placed in a test-tube one by ten centimeters; .1 cubic centimeter of concentrated sulphuric acid is added, and the tube is shaken vigorously. After thirty minutes one cubic centimeter of chloroform is added, and the tube is shaken just sufficiently to mix the solution, but not so as to disturb the sulphuric acid. Observations are made about thirty minutes later.

In order to determine the smallest quantity of cholesterol which would give a distinct reaction by this method, twenty-four test-tubes were prepared, containing six each of .00007, .00008, .00009, and .0001 gram of pure cholesterol dissolved in one cubic centimeter of chloroform. These were treated in the usual manner with .1 cubic centimeter of sulphuric acid and later with one cubic centimeter of chloroform. The stronger two solutions reacted perceptibly, but the reactions of the weaker two solutions were doubtful. The tubes were grouped in six sets, one solution of each strength in a set. Without knowing the strength of the solution in any tube, two observers arranged the four tubes of each set

according to the apparent depth of color. The results are shown in Table II.

TABLE II.

Observer A.				Observer B.			
0.0001	0.00008	0.00007	0.00009	0.0001	0.00008	0.00009	0.00007
0.0001	0.00009	0.00008	0.00007	0.0001	0.00009	0.00007	0.00008
0.0001	0.00009	0.00008	0.00007	0.0001	0.00009	0.00008	0.00007
0.0001	0.00009	0.00007	0.00008	0.0001	0.00009	0.00007	0.00008
0.0001	0.00009	0.00008	0.00007	0.00009	0.0001	0.00007	0.00008
0.00008	0.00009	0.0001	0.00007	0.00009	0.0001	0.00007	0.00008

It is clear from this table that solutions of .0001 and .00009 gram of cholesterol give a perceptibly stronger reaction than solutions of .00008 and .00007 gram give.

To ascertain the least perceptible difference between solutions, three sets of test-tubes were prepared, each set containing solutions which ranged from .00007 to .00015 gram, increasing by .00001 gram. One set served as a standard, and the tubes were arranged in serial order. Each of two observers prepared a similar set for the other, and each observer compared the unknown series with the known, looking through the tubes while holding them against a white surface. Three independent comparisons were made: first, twenty minutes after mixing with sulphuric acid, when the color of the solutions was yellow; second, thirty minutes after the addition of one cubic centimeter of chloroform; and third, ten minutes after the second comparison. The results of these observations are shown in Table III.

TABLE III.

Actual Quantities.	Apparently Equivalent Quantities Selected by Observer A.			Apparently Equivalent Quantities Selected by Observer B.		
	Before Addition of Chloroform.	30 Minutes After Addition of Chloroform.	40 Minutes After Addition of Chloroform.	Before Addition of Chloroform.	30 Minutes After Addition of Chloroform.	40 Minutes After Addition of Chloroform.
0.00015	0.00015	0.00015	0.00012	0.00015	—	0.00015
0.00014	—	0.00011	0.00014	0.00014	0.00015	0.00013
0.00013	0.00014	0.00012	0.00013	0.00013	0.00012	0.00014
0.00012	0.00013	0.0001	0.00011	0.00014	0.00015	0.00014
0.00011	0.00011	0.00014	0.00011	0.00011	0.00011	0.00012
0.0001	0.0001	0.00007	0.00011	0.00008	0.0001	0.00009
0.00009	0.00009	0.00007	0.0001	0.0001	0.0001	0.0001
0.00008	0.00008	0.00008	0.00007	0.00008	0.00007	0.00008
0.00007	0.00007	0.00008	0.00007	0.00008	0.00008	0.00007

It will readily be seen that a difference of .00001 gram cannot be detected by this method.

In another series of similar observations, solutions ranging from .00005 to .000425 gram, increasing by .000025 gram, were prepared and observed in the manner described above. The results given by the two observers are shown in Table IV.

TABLE IV.

Actual Quantities.	Apparently Equivalent Quantities Selected by Observer A.			Apparently Equivalent Quantities Selected by Observer B.			Maximum Error.	
	Before Addition of Chloroform.	30 Minutes after Addition of Chloroform.	40 Minutes after Addition of Chloroform.	Before Addition of Chloroform.	30 Minutes after Addition of Chloroform.	40 Minutes after Addition of Chloroform.	+	-
0.00005	0.00005	0.000075	0.00005	0.00005	0.0001	0.00005	0.00005	
0.000075	0.000075	0.0001	0.0001	0.000075	0.00005	—	0.000025	0.000025
0.0001	0.0001	0.0001	0.0001	0.0001	0.000075	0.000125	0.000025	0.000025
0.000125	0.000125	0.000125	0.000125	0.000125	0.000125	0.00015	0.000025	
0.00015	0.00015	0.00015	0.00015	0.00015	0.00015	0.00015	0	0
0.000175	0.000175	0.000175	0.000175	0.0002	0.000175	0.0002	0.000025	
0.0002	0.0002	0.0002	0.0002	0.000175	0.0002	0.0002	0.000025
0.000225	0.000225	0.0002	0.000225	0.000225	0.000225	0.000225	0.000025
0.00025	0.000275	0.00025	0.0003	0.000225	0.000275	0.000275	0.000075	
0.000275	0.000325	0.000275	0.000325	0.0003	0.0003	0.0003	0.00005	
0.0003	0.0003	0.000375	0.00035	0.000325	0.0003	0.000425	0.000125	
0.000325	0.00035	0.00035	0.000425	0.0004	0.000425	0.000425	0.0001	
0.00035	0.0004	0.00035	0.000425	0.00035	0.00035	0.000425	0.000075	
0.000375	0.000425	0.000425	0.000425	0.00035	0.000425	0.000425	0.00005	

It is clear from these results that within a certain range a difference of .00005 gram can readily be detected, and frequently a difference of .000025 gram.

The range most favorable for accurate differentiation is between .0001 and .00025 gram, just above the threshold of perception. This is in accordance with the psychophysics law known as Weber's law, which is thus described by Titchener²: "If we determine a series of just noticeable differences, in the middle region of the intensive scale, we find a very simple relation between change of sensation and increase of stimulus. At the beginning, where the stimuli are relatively weak, only a small addition is required to effect a noticeable increase in the intensity of sensation; as the series progresses, the additions become larger and larger; and towards the end, where the stimuli are relatively strong, the largest additions are needed."

In the tubes containing .00005 gram, the solution was colorless, but the sulphuric acid was slightly yellowish. Controls of sulphuric acid in pure chloroform gave no such reaction. It is possible that determinations even more accurate than these might be made by looking down into the tubes, as is done in ammonia determinations.

SUMMARY.

Satisfactory quantitative estimations of small quantities of cholesterol can be made by the following method :

The cholesterol is dissolved in pure chloroform (Merck Blue Label), and a series of dilutions is made which will bring at least one solution between .0001 and .00025 gram to one cubic centimeter of chloroform.

A set of tubes is made up, containing pure cholesterol in quantities ranging from .0001 to .00025 gram to one cubic centimeter of chloroform, each tube containing .000025 gram more than the preceding one.

To each tube is now added .1 cubic centimeter of concentrated sulphuric acid, and the mixture is thoroughly shaken. After thirty minutes one cubic centimeter of chloroform is added, and thirty minutes later the unknown solutions are compared with the known.

By this method differences of .00005 or .000025 gram can be detected.

This method is recommended especially for the estimation of cholesterol in the blood, lymph, and other fluids of which it is rarely possible to obtain a sufficient amount to enable one to isolate a weighable quantity.

[In making these observations I have been assisted by Dr. Grace Helen Kent, to whom I wish to acknowledge my indebtedness.]

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THE ANTISEPTIC AND BACTERICIDAL PROPERTIES OF EGG-WHITE.*

LEO F. RETTGER AND JOEL A. SPERRY.

(From the Sheffield Laboratory of Bacteriology and Hygiene, Yale University.)

As one of our most staple articles of food, the hen's egg has long been an object of unusual interest and study. The prevention of deterioration of eggs within the shell is in itself a problem of great economic importance. The question of the presence of bacteria in eggs that are fresh as well as those of different ages is one that has received much attention within the last few years, and regarding which there still is a wide difference of opinion. There is no doubt that eggs gradually deteriorate, even under the most favorable conditions of storage; but whether bacteria play a prominent rôle in the chemical changes that are brought about slowly is not so certain. It is not within the province of this paper to discuss these questions, but to present facts which have a more or less direct bearing on them.

It has been taken for granted that the only protection which an egg has against bacterial invasion and subsequent "spoiling" is the shell. That the shell serves as a good protective covering cannot be disputed, and may be explained largely by the fact that a normal egg is coated with a delicate layer of gelatinous substance. This coating is easily injured or destroyed when it comes in contact with water; hence the common belief that washing of eggs lessens their keeping qualities.

To the protective covering of the shell, and the shell itself, must be added another important agent in the prevention of bacterial invasion into the interior of the egg; namely, the antiseptic and bactericidal properties of the egg-white.

Different investigators have demonstrated the permeability of egg-shell to liquids and to bacteria. Zörkendörfer¹ observed that aqueous solutions of eosin readily pass through the shell. Also that bacteria penetrate the shell when the

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egg is allowed to remain for a short time in bouillon cultures of the bacteria. Wilm² found that the cholera vibrio passed through the shell in fifteen to sixteen hours when the egg was at least partly covered with a peptone culture of the organism. Eggs containing the cholera vibrio remained apparently normal for four or five days, after which they became turbid and gave off a hydrogen sulphide odor. *Bacillus coli* and certain other bacteria were also able to gain the interior of the eggs. Motility and size of the organisms determined their ability to pass through the shell.

Piorkowsky³ placed eggs in a tube or shallow dish containing peptone medium which had been inoculated on the previous day with *B. typhi*. The eggs were partly covered with the culture fluid for periods varying from twenty to ninety-six hours. The typhoid bacillus passed into the egg in twenty-four to forty-eight hours, temperatures of 28–37° C. being more favorable than 21°. According to Sachs-Mücke⁴ *B. dysenteriae* is unable to penetrate the shell of a normal egg. He found also that *Mucor corymbifer*, *Aspergillus niger*, *Penicillium glaucum* and *Penicillium brevicaulis* likewise fail to do so. He believes that the chance of infection is much greater in the oviduct than after the egg is laid.

Lange⁵ experimented with the following organisms: *B. coli communis*, *B. typhosus*, *B. paratyphosus*, *B. enteritidis* (Gärtner) and *B. botulinus*. After washing the eggs in corrosive sublimate, alcohol, and then ether, he placed them in bouillon cultures of the organisms and left them there for varying lengths of time, at 37° C. The passage of the bacilli into the interior of the eggs depended, in a measure at least, upon their motility. *B. typhosus* required two days to enter the white, and three days to enter the yolk. *B. coli communis* reached the white in one day, and the yolk in five. It is quite probable that the treatment of the eggs with corrosive sublimate, alcohol, and ether greatly facilitated the passage of the bacilli through the shell.

Eggs, both cooked and raw, have been employed as culture media for certain bacteria, especially the organism of Asiatic cholera (Hueppe, Zenthoffer, Bonhoff, Hammerl,

etc.). The employment of uncooked egg has, however, not been widely adopted, and the reason should appear quite obvious.

In a recent publication by Laschtschenko⁶ normal, undiluted egg-white is reputed to have marked antiseptic and bactericidal properties towards certain bacteria. He observed that when the whites of fresh eggs were kept in an open dish and constantly exposed they underwent no visible change aside from the rapid drying. Furthermore, they remained practically sterile. If, however, one-half to one cubic centimeter of nutrient bouillon was added to the albumen of one egg putrefactive changes took place in two to three days. The addition of a few drops of tap water to the white of an egg did not apparently lessen its resistance to the ordinary air and water bacteria; but when as much as two cubic centimeters of water were added the presence of certain organisms, particularly *B. fluorescens liquefaciens*, could be observed in three or four days.

A number of tests with pure cultures of *B. subtilis* showed that this organism was not only inhibited in its growth, but was destroyed in a very short time when exposed to the action of normal egg-white. Similar results were obtained with *B. megatherium*, *B. ramosus*, *B. anthracis*, *Proteus Zopfii*, and *Proteus Zenkeri*. Small amounts of water or nutrient bouillon did not materially lessen the bactericidal action. The addition of ten parts of water or saline solution to one part of albumen did not prevent the destruction of *B. subtilis*, and a dilution of the egg-white with fifty volumes of nutrient bouillon did not destroy the bactericidal properties of the white with reference to *B. Zopfii* and *Proteus Zenkeri*.

That the inimical action of the egg-white was due to a specific enzyme or anti-substance, and not alkali, or to the lack of favorable nutrient material, was shown by the heat test. Heating the white for thirty minutes at 65-70° C. destroyed the bactericidal properties.

By mixing the yolk with the white the germicidal action of the latter was greatly reduced. The mixture even served

as a favorable culture medium for bacteria, for example, *B. subtilis* and *B. anthracis*. These two organisms were found to multiply in the egg itself when introduced as far as the interior of the yolk.

In our investigation we have in a large measure repeated the work of Laschtschenko, and have endeavored to obtain additional data which might be of some interest and importance. Our work thus far has been done with egg-white and yolk after they were removed from the shell, and fully substantiates Laschtschenko's investigations. The organisms employed by us were *B. subtilis*, *B. cereus*, *B. megatherium*, *B. coli communis*, *B. typhosus*, *B. pullorum* (Rettger), *B. fluorescens liquefaciens* and non-liquefaciens, *Proteus vulgaris*, *P. mirabilis*, *P. Zenkeri*, *Staphylococcus pyogenes aureus*, and the obligate anaërobes, *B. putrificus* (Bienstock), and *B. edematis maligni*.

The following technic was used in our earlier tests with pure cultures of bacteria. Eggs which appeared to be normal and reasonably fresh were placed in fifty per cent alcohol and left there for ten minutes. They were then flamed (the small end) with a Bunsen burner, and a small portion of the shell cut out with sterile scissors. Five cubic centimeter quantities of the white and yolk were removed separately with a sterile pipette and placed in large test-tubes. The tubes were inoculated with one or more loopfuls of a bouillon culture of the organism in question. After shaking vigorously plates were poured at different intervals with one or more loopfuls of the inoculated white or yolk.

The technic was slightly improved upon before the work had continued very far. In the first place, broken glass was used to cut up the globulin of the egg-white in the test-tubes, and thus to facilitate the distribution of the bacteria in the tubes. Each tube was shaken vigorously, after which .25-.5 cubic centimeter of the inoculated egg-white were introduced into dilution flasks containing one hundred cubic centimeters of water, and the flasks shaken for thirty seconds. Agar plates were poured with .5 cubic centimeter of these dilutions. Examinations were always made in duplicate series, and the results averaged.

BACILLUS SUBTILIS, B. CEREUS, AND B. MEGATHERIUM.

Organism.	Immediately After Inoculation.	After 24 Hours.	After 72 Hours.
<i>B. subtilis</i>	200 colonies.	O	O
" "	o "	O	O
<i>B. cereus</i>	1,100 "	O	O
<i>B. megatherium</i>	o "	O	O
" "	o "	O	O

The germicidal action of egg-white on *B. subtilis*, *B. cereus*, and *B. megatherium* is strikingly shown in the above table. The time elapsing between the inoculation of the tubes and the pouring of the first sets of plates was five to fifteen minutes. *B. megatherium* was destroyed almost instantaneously in both experiments, so that no colonies were obtained in the first sets of plates. That the organism was present in the bouillon which was transferred to the albumen in the tubes was shown by its luxuriant growth when similar inoculations were made in the usual laboratory media. *B. subtilis* was also killed, in one of the experiments, before the first series of plates could be prepared. After twenty-four hours no growth was obtained in any of the plates.

B. COLI COMMUNIS AND B. TYPHOSUS.

Organism.	Immediately After Inoculation.	1 Hour.	24 Hours.	48 Hours.	72 Hours.	96 Hours.	5 Days.	1 Week.
<i>B. coli communis</i> . (N.Y.U.) (37°).	—	—	50	O	O	(Colonies	on plates.)	
<i>B. coli communis</i> . (N.Y.U.) (37°).	—	475,000	320,000	O	O	(Bacteria	per cub	licen-
<i>B. coli communis</i> . (N.Y.U.) (20°).	—	325,000	810,000	320,000	—	—	350,000	egg-wh
<i>B. coli communis</i> . (H.) (20°) . . .	1,000,000	—	575,000	—	360,000	—	—	ite.)
<i>B. typhosus</i> . (Y.M.S.) (37°).	725,000	—	525,000	O	—	—	—	
<i>B. typhosus</i> (W.) (37°)	430,000	—	550,000	—	—	3,000,000	—	

The organisms in this group are decidedly more resistant to the action of the egg-white than those in the preceding table. *B. coli communis*, strain N.Y.U. and *B. typhosus* Y.M.S., have been kept in the laboratory for at least seven years. Colon bacillus H. was isolated from hen's droppings within but a few weeks before the experiment, and *B. typhosus* W. was obtained from Washington about two years before. In all of the tests but one the numbers of the bacilli in the egg-white tubes were reduced, and in three instances the tubes were rendered sterile. The bactericidal action on *B. coli communis* appears to be stronger at 37° C. than at 20°. With *B. typhosus* W. there was merely an antiseptic action, as the number of bacilli increased from 430,000 to 3,000,000.

PROTEUS VULGARIS, P. MIRABILIS, AND P. ZENKERI.

Organisms.	Imme- diately After In- oculation.	1 Hour.	24 Hours.	30 Hours.	48 Hours.	72 Hours.	96 Hours.
<i>Proteus vulgaris</i> . (37°)	2,000,000	2,200,000	—	8,000,000	—	900,000	—
<i>P. mirabilis</i> . (37°) . .	250,000	—	500,000	—	—	9,000,000	—
<i>P. Zenkeri</i> . (37°) . .	0	—	0	—	—	—	—

Proteus vulgaris shows considerable resistance to the bactericidal action of the white, while *Proteus Zenkeri* is killed off immediately, or so soon that the plates must be prepared very quickly in order to obtain any colonies in them. There is a possibility, of course, that in this case too few of the bacilli were transferred to the egg-white from the bouillon culture, as there was no control experiment; the results agree very well, however, with those of Laschtschenko. The *Proteus mirabilis* strain used in this series of tests showed a high degree of resistance, although during the first twenty-four hours the number of bacilli was barely doubled.

STAPHYLOCOCCUS PYOGENES AUREUS, B. FLUORESCENS LIQUEFACIENS AND
B. FLUORESCENS NON-LIQUEFACIENS.

Organisms.	Imme- diately After In- oculation.	1 Hour.	24 Hours.	72 Hours.	96 Hours.	6 Days.
<i>Staphylococcus pyogenes aureus</i> (37°)	500,000	130,000	13,000	—	0
<i>B. fluorescens liquefaciens</i> (37°)	0	0	—	—	—
<i>B. fluorescens non-liquefaciens</i> (37°)	725,000	550,000	—	0	—

Incubation temperature undoubtedly has played no small part in the rapid destruction of the fluorescens organisms. Nevertheless, incubation alone does not bring about such results; these organisms grow quite luxuriantly at 37° C., though this temperature is far above their optimum.

BACTERIUM PULLORUM (RETTGER), STRAINS A AND E-4.

Organism.	Immediately After Inocu- lation.	24 Hours.	48 Hours.	72 Hours.	96 Hours.	120 Hours.	6 Days.	7 Days.	9 Days.
<i>B. pullorum</i> A (37° C.) .	900 (col.)	475	200	4	—	0	—	—	—
<i>B. pullorum</i> A (20° C.) .	1,400 "	150	60	} Same egg both g-w organi- isms.	—	—	—	—	—
<i>B. pullorum</i> E-4 (20° C.),	3,400 "	1,900	1,350		—	—	—	—	—
<i>B. pullorum</i> E-4	10,000 "	8,000	5,200		800	80	—	2	—
<i>B. pullorum</i> A (20° C.) }	1,000	—	300	—	110	—	—	—	3
<i>B. pullorum</i> E-4 (20° C.) }	Same egg-white. 5,000	—	4,500	—	4,000	—	—	—	3,000
<i>B. pullorum</i> A }	750	—	750	—	750	—	—	—	—
<i>B. pullorum</i> E-4 . . . }	Same egg-white. 3,000	—	3,000	—	3,000	—	—	—	—
<i>B. pullorum</i> E-4 (20° C.) }	4,200	4,000	—	—	2,500	—	—	—	—
<i>B. pullorum</i> E-4 (37° C.) }	Same egg-white. 4,200	1,200	—	—	3	—	—	—	—

B. pullorum A was an old strain obtained six years ago from the blood of a chick which had died of bacillary white diarrhea. E-4 was procured from infected ova (hen) within less than a year of the time of these experiments. Feeding experiments conducted on a large scale with young chicks

demonstrated the fact that A had lost entirely its ability to reproduce the disease, white diarrhea, while E-4 still possessed a high degree of virulence.

In the above table the two strains of *B. pullorum* are seen to show a striking difference in their resistance to the bactericidal action of egg-white, and it is of particular interest to observe that the virulent strain is the more resistant one. In one instance the egg-white had no destructive action on either, but merely prevented development. This must be explained on the hypothesis that the white in this case was less active than in the other experiments, and therefore affected both strains alike.

The last two series of results seem to indicate that the bactericidal action of the egg-white is accelerated with increased temperature.

In order to demonstrate whether the inability of the bacteria to multiply or even to remain alive was due merely to a lack of necessary nutritive material in the egg-white, or certain physical properties, or to so-called anti-substances, the following tests were made: Tubes of the egg-white were inoculated with *B. pullorum* A and E-4; two with A and two with E-4. One tube of each was heated at 65°-70° C. for fifteen minutes; the other was left unheated. Agar plates were poured immediately and at definite intervals thereafter.

INFLUENCE OF HEAT ON THE BACTERICIDAL PROPERTIES.

Organisms.	Immedi- ately After Inocula- tion.	24 Hours.	48 Hours.	92 Hours.	140 Hours.
Unheated egg-white:					
<i>B. pullorum</i> A (37°) . . .	500 (col.)	400	57	0	0
<i>B. pullorum</i> E-4 (37°) . .	1,100 "	1,100	1,000	900	350
Egg-white heated at 65-70° C. for 15 minutes:					
<i>B. pullorum</i> A (37°) . . .	500 "	At end of distingui sh.	24 hours col	onies too nu	merous to
<i>B. pullorum</i> E-4 (37°) . .	1,000 "	At end of distingui sh.	24 hours col	onies too nu	merous to

These results are in accord with those of Laschtschenko. The bactericidal properties are destroyed at a temperature varying from 65 to 70° C. Egg-white which has been heated at this temperature serves as a good culture medium.

PUTREFACTIVE ANAËROBES.

The putrefying organisms employed were *B. putrificus* and *B. edematis maligni*. Five cubic centimeter quantities of egg-white in sterile test-tubes were liberally inoculated with these organisms. The transfers were made from tubes of egg-meat media which had undergone marked putrefaction. At the same time that the egg-white tubes were inoculated with the anaërobes, similar transfers were made to dextrose-bouillon, egg-yolk, and egg-meat. The tubes were rendered anaërobic by the Wright (pyrogallol) method, and incubated for a week or more at 37° C. Putrefactive decomposition made itself apparent very soon in the yolk and in the egg-meat medium, and the dextrose-bouillon became clouded in three or four days. In the egg-white tubes there were no signs of change in the character of the white except a slight discoloration. The tubes remained clear, and there was not the slightest odor of putrefaction or other decomposition. Microscopic examination failed to reveal the presence of any bacteria whatever; whereas, in the yolk and the egg-meat tubes the organisms in question were found to be present in large numbers.

As the work upon which this paper is based will be continued for some time, we are loathe to offer any explanations at present of the pronounced antiseptic and bactericidal properties of egg-white towards certain bacteria. Whether there is present some anti-substance which bears a close analogy to those in the blood and other animal tissues is in itself a problem of much interest. The possibility that pure unchanged proteins may in a measure play the part of an antiseptic or disinfectant is not a remote one. Such a thing is suggested indeed by the results of the recent investigations of Bainbridge,⁷ who demonstrated that certain organisms

(*B. typhosus*, *B. coli communis*, *B. pyocyaneus* and *Staphylococcus pyogenes aureus*) do not appreciably break down pure or unchanged egg-albumin or serum-protein, even in the presence of sufficient non-protein nitrogenous food to insure vigorous bacterial growth.

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THE FORMATION OF PIGMENT BY THE DERMAL CHROMATOPHORES.*

OSCAR T. SCHULTZ, M.D.

*(From the Pathological Laboratory of Western Reserve University,
Cleveland, Ohio.)*

The purpose of this communication is to report the result of an investigation into the functional activity, as expressed by morphological changes, of a particular type of cell — the chromatophore of the skin. While there is some risk of drawing false conclusions in explaining certain morphological changes as the result of cellular physiology, normal or abnormal, some fundamental facts have been established by the cytologist and it is attempted here to correlate what occurs in a specialized variety of cell with what has been found to hold for other species of cells.

The material (obtained through the courtesy of Professor W. T. Corlett) upon which the study is based is skin removed from a case in which the clinical diagnosis was mycosis fungoides. In addition to the fungating tumor-like areas which occur in this disease the skin of the entire body showed a mottled brownish pigmentation. Upon a deep brown background were scattered irregularly-shaped areas of nonpigmented skin, one to several millimeters in diameter. Where the formation of pigment was more recent, where the discoloration was advancing, that is, at the margins of the unpigmented spots, the skin had a reddish tint, the color being a mahogany brown rather than the deeper, duller brown of the older areas. This is not the place for a detailed discussion of the pathological histology of the condition to which dermatologists have applied the terms mycosis fungoides or diffuse sarcomatosis. In brief, the tissues examined show a process which is of a diffuse, sub-acute, inflammatory nature. There is nothing, in material removed upon several occasions at intervals of four to six weeks between removal, to warrant the assumption that in

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the present case there was anything akin to sarcomatosis. At the present time we are concerned only with the pigment producing cells of the skin. While the inflammatory process is most intense in the tumor-like areas, the less involved pigmented skin also gives evidence of a mild, diffuse, inflammatory process. I have no desire to add anything, one way or the other, to the controversy as to whether any of the pigment present in normal skin is actually produced by the epidermal cells in which it is found. In my material the increased pathological pigmentation is due to an increase in the number of chromatophores in the subepidermal tissues. These cells have reacted to the inflammatory stimulus by proliferation just as have the other elements, connective tissue and endothelium, present in the same region. The cells of the stratum mucosum of the epidermis show no appreciable increase in pigment content. This is probably to be explained by the fact that, because of the diffuse character of the inflammation, the chromatophores are more deeply situated than normal. Only very few are present immediately beneath the epidermis, so that the pigment is not readily transferred to the cells of the latter. Because the chromatophores are reacting by moderately rapid proliferation, as are the other cells of the cutis, to what appears to be a relatively mild inflammatory stimulus, the material has been especially fortunate. Several stages of the life history of the pigment-producing cell are present in every field, so that the cycle of the cell from youth to old age can be more readily studied than is the case in the slow multiplication of the normal skin on the one hand or the abnormally rapid multiplication with its many disturbing factors in the malignant pigment cell tumors on the other.

It is not necessary to review the differences in opinion which have existed and which, perhaps, still exist as to the nature of the pigment cell. Marchand and Ziegler, among pathologists, have been strong in their opposition to the idea, prevailing in pathological literature, that the chromatophore differs from the ordinary connective tissue cell only in the pigmentation of its protoplasm.

Schneider (1902) in his very exhaustive *Lehrbuch der vergleichenden Histologie der Thiere* gives the chromocyte only the merest mention in speaking of cells whose protoplasm shows granules. Prenant (1904) considers the pigment producing cells to be specialized cells. That the chromatophore present in the cutis beneath the epidermis — the pigment cell which alone is the subject of this paper — is of mesoblastic origin is so evident as to require no proof. It differs morphologically, however, from the mesodermal connective tissue cells among which it lies in its non-formation of fibrils. In young and adult pigment cells in normal, inflammatory and tumor material I have never been able to demonstrate fibroglia fibrils by the use of Mallory's staining methods. That the dermal chromatophore differs greatly from the connective tissue cell in the development associated with a highly specialized function is the chief point that I wish to establish.

As to the chemical nature of the pigment little enough is known. Concerning the mode of its formation divergent opinions have been held. List (1889) and Ehrmann (1885, 1886, 1892, 1896) have stated that the pigment is derived directly from the hemoglobin; Jarisch (1891) that it is a protoplasmic product, not derived from the blood and Kromayer (1893, 1897) that it is formed by a degeneration of protoplasmic fibrillæ, a spongioplasm. That it is certainly not derived from the hemoglobin of the blood, that it is not a product of the cytoplasm alone and that it is not a degeneration in the ordinary sense of that term I hope to show.

Leaving out of consideration the cells whose normal function it is to produce pigment, something has been added to our knowledge of pigment formation by cytological studies. In the heliozoön (*Actinosphaerium eichhorni*), in the condition of depression into which this multinucleated protozoön falls under certain conditions, R. Hertwig (1904) described the transformation of chromatin into pigment. It is useless, excessive chromatin which has been cast out into the cytoplasm which is thus changed. Only a small proportion of the extruded nuclear material is so altered. Howard

(1908a), working upon the same animal under the same conditions, also noted the transformation of extranuclear chromatin into pigment and was, furthermore, able to detect the same change within the hypertrophied and hyperchromatic nuclei themselves. Rössle (1904) described the transformation of extranuclear chromatin into pigment in the cells of melanomata. Howard and I (1911) have seen a similar process, in very rare instances, in tumors derived from nonpigmented cells. In *Actinosphaerium eichhorni*, in which the derivation of pigment from chromatin is best established, the process is a purely degenerative one. The pigment comes from chromatin which has been extruded from the nucleus for the purpose of decreasing the marked hyperchromatism present in the depressed organisms. The greater portion of the chromatin is broken down by the cytoplasm or cast out from the latter. Only a small portion, not broken down or extruded rapidly enough, is transformed into pigment, which in its turn is extruded. A similar derivation of pigment occurs in the dermal chromatophores. But here the process is not a degenerative one — the chromatophore is a highly differentiated cell whose normal function is the formation of pigment; the process is the result of specialized physiological activity.

For an interpretation of the series of changes through which the dermal chromatophore passes in the cycle from its infancy to its senility there is necessary some understanding of R. Hertwig's nucleus-plasma relation doctrine, a doctrine of the most fundamental importance in cellular physiology. The facts upon which this theory are based have recently been reviewed by Howard (1908b), so that a restatement is not necessary here. The most important and essential feature of the nucleus-plasma relation is the definite volume relationship which exists for every species of cell, a relationship which may vary within physiological limits in various conditions of growth and activity of the cell. This basic principle has most widespread application. A necessary corollary of the doctrine and one of chief importance for our present purpose is the proof offered by it of the

mutual interdependence of nucleus and cytoplasm and of the consequent participation of the nucleus in cellular activity. A further point brought out by R. Hertwig was the extrusion of chromatin from the nucleus into the cytoplasm in experimentally depressed protozoa, the purpose of this phenomenon being a restoration of the normal nucleus-plasma balance by relieving the hypertrophied nucleus of some of its superabundant material. These extruded chromatin granules he termed chromidia. Our knowledge of chromidia and of their varying nature, fate, and function has been greatly widened since R. Hertwig's earliest observations. The chromidia of R. Hertwig were chromatin granules and masses present in the cytoplasm, which had been cast out from the nucleus to decrease the nuclear volume and to prevent fatal depression; arrived in the cytoplasm they were of no functional value to the cell. Extranuclear chromatin becomes a much more important cell constituent in Goldschmidt's (1904) idea of the chromidial apparatus. In very highly active cells the nuclear derivatives normally present in the cytoplasm are not excessive chromatin which is merely to be gotten rid of, but constitute an element of the cell essential for the specialized activity of the cell. The chromidial apparatus of Goldschmidt is as truly a cellular organelle as is the locomotory apparatus of a protozoön and for certain cells has a characteristic morphology. The Nissl granules of the nervous system ganglion cells constitute the most familiar example of functional extranuclear chromatin, an example of a chromidial apparatus of quite characteristic morphology.

Goldschmidt's conclusions were based largely upon a study of the muscle cells of a species of *Ascaris*. He showed that during rest a chromidial net is formed by the passage of chromatin from the nucleus into the cytoplasm and that this extranuclear material is used up during the activity of the muscle cell, to be again replaced during the next resting period. Dolley (1909a, 1909b, 1910) has established that a similar process occurs in the ganglion cell. He has described the formation of a chromidial apparatus

(storing up of Nissl substance) during rest, its partial consumption during normal activity, its disappearance and secondary replacement during activity leading to fatigue and the practically complete consumption of both extra- and intra- nuclear chromatin in the continued over-activity which leads to the exhaustion and death of the cell.

The formation of secretion products from nuclear materials has been noted by a number of observers in metazoan gland cells and in secreting plant cells. In such cells the extranuclear material constitutes a chromidial apparatus.

In the dermal chromatophores nuclear derivatives enter directly into the formation of pigment. It is upon the nuclear materials that the specialized function of the cell is dependent. The process is identical with that which takes place in gland, muscle, and ganglion cells — the participation of the nucleus in specialized cell function by a transformation of nuclear derivatives into the products or physiological activities characteristic of the cell. But the chromatophore differs from Goldschmidt's muscle cell and Dolley's ganglion cell in that in both the latter cellular activity is intermittent, whereas in the pigment cell the formation of its characteristic product is a continuous process. Changes do not follow by alternate stages, as in muscle and ganglion cells, but by a gradual and continuous succession of events which begins in the cell's infancy and stops with its senility.

In the participation of the nucleus in cellular activity the nucleolar substance takes an important part. According to R. Hertwig (1902) the chief function of this material is the organization of chromatin by uniting to itself substances derived from the cytoplasm. When, after having been extruded from the nucleus into the cytoplasm, the chromatin breaks down, the characteristic basic staining reaction disappears and gives way to the acid staining property of the nucleolar substance. This change is well seen in the karyorrhexis associated with necrosis. It occurs in the chromatophore when chromatin is transformed into pigment.

The description which follows is based largely upon

Zenker fixed material stained with eosin and Unna's alkaline methylene blue. The changes noted are considered stages in a chain of events. The definite and orderly sequence of these stages constitutes the life cycle of the chromatophore. That they follow each other in the order indicated cannot be proven with absolute certainty from a study of fixed material alone. But the sequence is such as conforms to established cytological facts — the latter do not permit any other interpretation.

The earliest stage in the life cycle of the chromatophore is, necessarily, one which cannot be recognized with certainty. It is an undifferentiated mesoblastic cell, differing little or not at all from the other mesodermal cells (fibroblasts and endothelial cells) present. The finely meshed cytoplasm constitutes a quite considerable proportion of the cell volume. The nucleus is large, vesicular, with most of its chromatin in the form of very fine granules scattered about upon a linen reticulum whose meshes are somewhat coarser than those of the cytoplasm. In addition to the finely granular chromatin one, rarely two to four, larger chromatin masses may be present.

A little later the cells begin to differentiate. They begin to take on certain morphological characteristics which lead up to those that distinguish the adult cell. The cell body is elongated and spindle shaped. Such cells are much like young connective tissue cells in which fibril formation is just beginning. In the differentiating chromatophore, however, the nucleus is, as a rule, broader than that of a spindle connective tissue cell of the same size. More important than the change in shape which occurs at this time is the alteration in the cytoplasm. The latter has a diffuse bluish color, due to the presence of minute chromatin granules. Frequently these granules form a fine but quite definite cytoplasmic meshwork. Cells with such an early chromidial net may already show the beginning of the branching which becomes so marked in the adult cell. In this stage the nucleus is still vesicular, a trifle smaller than that of the undifferentiated cell and with a chromatin content at least

equal to that of the earliest stages noted. The fact that the chromatin content of the nucleus has not appreciably decreased, in spite of the amount of chromatin which has been given off to the cytoplasm, is evidence of continued nuclear activity. Chromatin formation upon the part of the nucleus must at least equal the transferral of nuclear material to the cytoplasm.

There are present in small numbers cells which have not yet begun to show any change in shape, but whose cytoplasm contains relatively large chromatin clumps. It is impossible to say whether such cells are an intermediate stage between the undifferentiated cell and the spindle or branched cell with a fine chromidial network or whether they are examples of a disturbance of the normal course of events. They are not numerous, so that they would not seem to be a normal intermediate stage. On the other hand, there is evidence, to be noted later, that the normal process of differentiation may become upset.

After the cell has elongated and chromatin has made its appearance in the cytoplasm there occur the changes which are more directly associated with the formation of pigment. The cytoplasm has a decided violet tinge. This color is due to the presence of innumerable minute chromatin granules, like those already noted in an earlier stage, and many granules of varying but somewhat larger size which stain deeply with eosin. Bearing in mind the relation of chromatin and nucleolar substance to each other, the red stained granules are the older and have been formed by a fusion of extra-nuclear chromatin granules, the basic staining property disappearing with the increase in age. Brown pigment granules, considerably larger than the red stained ones, are already present, and there are seen all gradations in size and color from small red granules to large brown ones. The nucleus retains its original size but is loaded with chromatin. The latter material is being constantly formed by the nucleus in an amount even greater than that transferred to the cytoplasm to be here transformed into nucleolar substance and

then into pigment. Cells of the kind described bear a striking resemblance to very active gland cells. The similarity is heightened by the actual extrusion of pigment granules from the cytoplasm. The cells are arranged with their long axes parallel to the surface of the skin and the pigment extrusion is always much more marked on the side toward the epidermal surface.

Having passed through the very active adolescent stage the cell approaches maturity. The activity of the nucleus decreases. Chromatin extrusion has exceeded chromatin formation. The nucleus loses the hyperchromatism which characterizes the previous very active stage and the amount of chromatin in the cytoplasm is small. Most of the cytoplasmic chromatin has been transformed into nucleolar substance and much of the latter has been changed into pigment. The cell is now greatly elongated with very fine processes.

Although the transference of nuclear materials to the cytoplasm has ceased, the transformation of the nuclear derivatives already present outside the nucleus continues until practically all the granules which in the earlier stage stained red become changed into pigment. Where the cytoplasm can be seen between the brown pigment granules it has the finely meshed network and the faint pink color of undifferentiated protoplasm. The nucleus is vesicular, well rounded, and apparently rich in fluid.

With the cessation of activity the cell passes into a resting condition of senescence. It now shows the multiple branching so characteristic of the chromatophore of the normal skin. The cytoplasm is densely packed with dark brown granules of varying size. The nucleus has lost fluid, is irregular in shape and is so condensed and so deeply stained that a reticulum cannot be seen. In most cells it is lobulated; often it is broken into two or a small number of fragments. The lobulation and fragmentation are not to be confounded with the karyorrhexis of a dead cell. They are evidence, not of the death, but of the old age of the nucleus, just as similar changes in the nuclei of polymorphonuclear leucocytes and other cells indicate senility.

Mention was made above of disturbances in the normal sequence of events. Certain cells show changes which indicate that differentiation of cytoplasmic structure may be premature, that it may outrun the differentiation of cell shape. As a possible example of such a process attention was called to the irregularly polyhedral cells which contain relatively large chromatin masses. Other cells, present in fair numbers, show a cytoplasm rich in fine chromatin granules, but with the spheroidal or polyhedral shape of undifferentiated cells. The cell body is that of an embryonic cell, the cytoplasmic structure that of an older cell which has become spindle shaped or branched. The nucleus may be somewhat indented. In such cells the nuclear derivatives present in the cytoplasm undergo the same changes as in the normal cycle. Granules which take the stain of nuclear substance and others with the brown color of finished pigment make their appearance. In this way there may be produced spheroidal cells filled with brown pigment, that is, cells which have not undergone the normal differentiation in shape but in which the cytoplasmic elements have run through their entire sequence of changes. Spheroidal cells deeply laden with pigment occur in great numbers in rapidly growing melanomata. Since the chromatophores of many lower animals are contractile the same property has been given as the explanation of the spheroidal cells of tumors. Until active contractility of the pigment cell of the human skin is proven it seems unnecessary to fall back upon such a purely theoretical property to explain the spheroidal adult cells present in pigmented tumors. In the tumor cell the growth rate is much more rapid than normal. Nuclear materials are extruded in abnormal amounts and are transformed into pigment before there is time for the cells to take on the normal elongated and branched shape.

In the diffuse, subacute, inflammatory process which has involved the skin the chromatophores have reacted by proliferation. In the undifferentiated mesoblastic cells mitoses are seen. Just what proportion of such cells in indirect division are young chromatophores cannot be determined

because the cell has not the morphological characteristics which permit its certain recognition. Even if the majority of the cells in mitosis were chromatophores they are not numerous enough to account for the number of adult and senile cells which have been produced. In a considerable number of cells which already show the differentiation of cytoplasmic structure characteristic of the developing chromatocyte two nuclei are present. Earlier stages which indicate a direct nuclear division are also seen. In tumor cells amitosis occurs with such frequency as to indicate that it is an important factor in the very active proliferation. In the increased multiplication of the chromatophores in the inflammatory condition studied direct division appears to predominate over mitosis.

The young pigment cells produced by the inflammatory proliferation must have come originally from the senile chromatophores present in the normal skin. Nothing has been seen, however, to indicate through what stages the senile cells pass to render them capable of division. Certain it is that the senile cell, as such, is incapable of multiplication. The most probable explanation of what occurs is the loss of the pigmented, completely differentiated cytoplasm and a reorganization of the senile nucleus by the taking up of fluid. This nuclear reorganization is followed by a period of growth upon the part of both the nucleus and the small amount of cytoplasm surrounding it, leading to the formation of an undifferentiated cell of the mesoblastic type. Such cells are capable of mitosis before differentiation begins and of direct division after the process of differentiation has been initiated.

SUMMARY.

In the skin from a case of almost universal pigmentation of inflammatory origin the subepidermal chromatophores are greatly increased in number.

The proliferating pigment cells show changes indicative of a high degree of physiological specialization.

In the beginning of the process of differentiation chromatin is thrown out of the nucleus into the cytoplasm, leading to the formation of a functional chromidial net.

The chromatin present in the cytoplasm becomes changed into a material which has the staining reactions of nucleolar substance. Further change leads to the transformation of this material into pigment.

The pigment produced is derived directly from nuclear materials. It is probable that in this transformation specialized activity, chemical or catalytic, upon the part of the cytoplasm also takes part, since in other cells in which active chromidiosis occurs none or only a very small proportion of the extruded chromatin is transformed into pigment.

The pigment produced by the pigment forming cells of the skin is not a hemoglobin derivative and it is not a degeneration in the ordinary sense. It is the product of the specialized physiological activity of the cell by which nuclear derivatives are transformed directly into pigment.

In the early stages of differentiation the chromatophores may divide by amitosis.

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A STUDY OF THE RENAL EPITHELIUM IN VARIOUS TYPES
OF ACUTE EXPERIMENTAL NEPHRITIS AND OF THE
RELATION WHICH EXISTS BETWEEN THE EPITHELIAL
CHANGES AND THE TOTAL OUTPUT OF URINE.*

WM. DE B. MACNIDER, M.D.

(*From the Laboratory of Pharmacology of the University of North Carolina.*)

SYNOPSIS.

INTRODUCTION.

REVIEW OF THE LITERATURE.

SOURCE OF MATERIAL: Kidney poisons. Dosage. Technical methods employed.

THE RELATION WHICH EXISTS IN THE DIFFERENT TYPES OF NEPHRITIS BETWEEN THE EPITHELIAL CHANGES AND THE TOTAL OUTPUT OF URINE.

SUMMARY: The relative affinity of the various poisons for the epithelial and for the vascular elements of the kidney.

CONCLUSIONS.

BIBLIOGRAPHY.

INTRODUCTION. — In a review of the exhaustive literature which has developed concerning acute nephritis one is struck by the disproportion which exists in the various experimental and clinical observations between the relative importance that is attributed to the vascular and to the epithelial changes in leading to a condition of anuria. This is true whether the subject be approached from the experimental or from the clinical side.

It is, of course, realized that both elements of the kidney participate in the development of such a condition; and yet almost without exception the vascular reaction has received, both from the experimental and from the clinical side, the major consideration and the epithelial changes have been relegated to a place of minor importance, so far as these changes are concerned, in influencing the total output of urine.

Whether or not at the present time we agree with the statement of Cohnheim,¹ that "it need hardly be added that cloudy swelling and fatty degeneration of the tubular

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epithelium can never cause even the slightest resistance to the passage of urine," and no matter what theory or combination of theories we hold concerning the formation of urine, one fact is certainly established that whatever urine there is formed makes its exit from the kidney by passing through tubules lined by epithelium, and that this epithelium colloidal in nature is competent to swell and partly or completely block the channel through which the urine is passing.

While the epithelial element of the kidney will be principally studied in the pathological reaction obtained in various acute experimental nephritides, it is not the intention of this investigation to minimize the part played either in an anatomical or in a physiological way by the vascular changes developing during the same reaction.

Again, the present study has been conducted primarily from an anatomical standpoint, and it may be questioned whether or not it is justifiable to make physiological deductions from anatomical alterations. It is fully realized that a real danger may be encountered here, but if there be a constant association, especially if the environment differs, of anatomical changes with physiological alterations, this element of constancy tends to minimize the danger. Even though in the case of the kidney this statement be true, and granting that the association of anatomical changes be constant, such an association does not conclusively differentiate, as is the case in this investigation, which one of the kidney elements, epithelial or vascular, is principally concerned in reducing the output of urine or in causing an anuria. This important part of the investigation dealing primarily with the physiological reaction of the kidney in experimental nephritis, in which either the vascular or epithelial element of the kidney is principally involved, has formed the basis of another investigation. The deductions which are allowable from this standpoint substantiate the deductions which will be made from the following anatomical data.

The present investigation has for its object a study of the renal epithelium in various types of acute experimental

nephritis with the object in view of determining to what extent, if any, the total output of urine may be correlated with changes in the renal epithelium, especially those of an obstructive nature.

Review of the literature. — The following review of the literature dealing with the subject under discussion is purposely limited. Those contributions on acute nephritis have been selected which have some definite bearing upon the subject of this paper, *e.g.*, the epithelial changes. The experimental investigations have been selected with the exclusion of clinical observations even though the latter are of great value. The principal reason for such an exclusion is that the clinical deductions relative to the kidney pathology are very frequently not substantiated by post-mortem examination, and without such a control the element of surmise is so great that it has seemed advisable to exclude such observations.

The two most important of recent contributions to the study of experimental acute nephritis are those by Schlayer and Hedinger,² and by Pearce, Hill, and Eisenbrey.³ The latter investigation was modelled to a considerable extent after the first one, but differs in several particulars and especially in the type of animal in which the various reactions were studied. The former investigators employed exclusively the rabbit, the latter the dog.

The primary object of the investigation by Schlayer and Hedinger was to determine the difference in the vascular reaction in the two main types of nephritis, *e.g.*, the glomerular and the tubular. For their studies in the vascular type they employed as kidney poisons, cantharidin, arsenic and diphtheria toxin, and for the tubular type potassium chromate and corrosive sublimate.

In the early stages of the nephritis induced by the chromate and by mercury they found that the animal eliminated a larger amount of water; while in the cantharidin animals the initial polyuria was absent. As the cantharidin nephritis progressed, or if much cantharidin was primarily employed

in the production of the nephritis, the diuresis was slight or wholly absent.

The initial diuretic effect of various renal irritants has been very frequently noted. As will be later indicated, this reaction on the part of the kidney has developed rather constantly in the series of animals employed in this investigation.

Austin and Eisenbrey⁴ noted the occurrence of diuresis in nephritis due to small doses of uranium and cantharidin and also noted a transient increase in the elimination of chlorides at the same time that the output of urine was increased.

Weber,⁵ working with chromate nephritis, noted an increased flow of urine in the early stages, the severer grades of the nephritis being accompanied by an anuria which was uninfluenced by theocin.

Helen and Spiro⁶ observed in the glomerular nephritis produced by cantharidin that the excretion of water was greatly lessened, and that the diuretic effect of fluids, saline diuretics, caffeine, and phlorizin was diminished or abolished, and also that those nephritic poisons which acted on the tubular epithelium had less effect in increasing the output of urine. This last statement is certainly in accord with the results usually obtained from using tubular poisons.

Ruschaupt,⁷ employing the chromates in rabbits in small doses, obtained a slight diuresis; while Cohnstein,⁸ Vejux-Tyrode and Nelson⁹ were able to produce diuresis in rabbits by the use of preparations of mercury.

In the above mentioned instances in which preparations of mercury were employed, these substances were used primarily in a study of their diuretic effect and not as renal poisons. It is likely that diuresis induced by such substances borders very closely upon a beginning nephritis, glomerular in character. The same statement would apply to the diuresis induced by such irritants as turpentine.

The investigation of Pearce, Hill, and Eisenbrey³ is principally concerned with the vascular reaction in acute nephritis. It takes into consideration in some detail an account of the epithelial alterations. The authors were able to distinguish types of nephritis in which either the tubular or the vascular

changes predominated. They were not able to conclude, however, that a given poison produced an exclusively tubular or vascular injury. Potassium chromate, corrosive sublimate and uranium nitrate caused extensive tubular injury and in the early stages of the nephritis showed no evidence of vascular injury, except physiologically. When such methods were employed they were able to demonstrate in the early stages an exaggerated contraction and dilatation of the vessels and also an increased diuresis.

Arsenic and cantharidin acted as vascular poisons and produced but little injury to the tubules. Both of these poisons tended to cause an anuria, which was characterized by minimal contraction and dilatation of the renal vessels and little or no flow of urine.

Finally in this investigation two types of late tubular nephritis are described: one anuric and accompanied by gastro-intestinal symptoms and the other polyuric until the time of anesthesia.

In the later investigation by Pearce and Eisenbrei,¹⁰ which consists in a physiological study of experimental nephritis due to bacterial poisons and cytotoxic sera, the authors were again able to show that different poisons affected different kidney structures. In addition to this, they were able to show that in a nephritis with little or no anatomical evidence of vascular injury, physiologically the vascular incompetency might be pronounced, and conversely that in a nephritis with anatomical evidence of vascular injury the physiological reaction might be negative.

Pearce,¹¹ in his Harvey lecture on "The Problems of Experimental Nephritis," furnishes a classification of the usually employed kidney poisons, based on their predominating affinity for the vascular or the epithelial elements of the kidney.

Sollmann,¹² in an extensive review of the drugs irritant to the kidney, notes that all of the metals so far as they have been studied cause a nephritis when absorbed into the body.

In addition to the investigations which have been mentioned,

that deal with different types of acute experimental nephritis, the more important researches which have been concerned with the pathological manifestations of one type, are as follows:

Uranium nephritis. — Heineke and Myerstein,¹² employing uranium nitrate, were able to demonstrate a definite vascular disturbance in addition to a pronounced action upon the renal epithelium; while Dickson,¹⁴ in an extensive series of experiments in which the guinea-pig was the animal employed, came to the same conclusions.

Christian,¹⁵ in his work on uranium nephritis, in which the vascular reaction was studied, observed oval or irregular homogeneous droplets .5-4 microns in diameter in the wall of the capillaries of the glomerulus. This observation is of special interest on account of the rarity with which distinct histological transformations have been observed in the glomerular vessels.

The work of Schirokauer,¹⁶ on the uranium nephritis in rabbits is of interest on account of the associated anasarca. The development of this condition he attributes to the retention of salts, especially the chlorides.

Cantharidin nephritis. — The literature on cantharidin nephritis is perhaps the most extensive of that of any of the kidney poisons. Only the more important investigations will be reviewed here.

The experiments and deductions of Helen and Spiro in this type of nephritis have been previously mentioned.

Richter and Roth¹⁷ in their work on cantharidin nephritis were able to show that small doses of cantharidin acted almost entirely on the glomeruli. This action consisted in a dilatation of the glomerular vessels with a leucocytic infiltration of Bowman's capsule. With the small doses the quantity of urine was increased; while with the larger doses it was diminished. The epithelium of the convoluted tubules was involved by the larger doses, and this late in the nephritis. Lyon¹⁸ described similar epithelial changes to those observed by Richter and Roth and laid special stress upon the necrosis of the epithelium produced by cantharidin in the ascending loops of Henle. Bradford,¹⁹ noting the selective action of nephrotoxic substances, lays stress upon the glomerular nephritis induced by cantharidin.

These apparently contradictory results concerning the affinity of cantharidin for the different renal tissues can in great measure be reconciled when the quantity of the substance employed is considered and when the duration of the nephritis is taken into account. If a small quantity of cantharidin be employed subcutaneously, and the resulting nephritis be terminated early, the glomerular lesion in the great majority of instances predominates rather conspicuously. On the other hand if larger doses of cantharidin be employed, or if small doses be employed and the nephritis be allowed to continue for some days, the epithelial pathology is far more

striking than is the vascular pathology. This is especially true of the glomeruli.

Arsenical nephritis. — The principal observations concerning the nephrotoxic action of arsenic have been made by Helen and Spiro,⁶ and by Pearce, Hill and Eisenbrey.³ In the first mentioned investigation the authors consider that arsenic causes a specific paralysis of the capillaries which is most pronounced in the glomerular vessels. The space in Bowman's membrane may be obliterated by the distended vessels. The epithelium of the convoluted tubules was affected in various degrees, while the epithelium of the straight tubules was but slightly involved.

In the latter investigation arsenic finds a classification as a vascular poison along with cantharidin, and the infrequency with which the epithelium is attacked is mentioned.

Chromate nephritis. — Concerning the effect of the chromates as kidney poisons there is more unanimity of opinion than is the case with any of the other nephrotoxic substances. Though they differ in some minor details, especially as regards the vascular reaction from this group of poisons, the principal investigations in which the chromates have been employed show them to have a marked predilection for the renal epithelium. This action has been clearly shown by the work of Helen and Spiro,⁶ Austin and Eisenbrey,⁴ and Ruschaupt.⁷

Belonging to the same group of substances, so far as their toxic effect is concerned, are bichloride of mercury and aloin.

In addition to the observations made by Pearce on the toxic effect of bichloride of mercury on the kidney, Kobert³⁰ has shown that in addition to the usual effect mercury produces an occlusion of the renal tubules by calcareous deposits.

Murset,³¹ in 1885, while employing aloin as a kidney poison, observed that it produced a degeneration of the epithelium of the convoluted tubules with very little effect on the vascular side of the kidney. In such a nephritis the output of urine varied. In some instances it was increased, while in others it was diminished.

In addition to the anatomical studies of the kidney which have been made during its reaction to various nephrotoxic substances, the renal epithelium has been studied during a diuresis from various drugs and when subjected to the action of solutions of various salts in different molecular concentrations.

In this connection V. Sobieranski,³² and Archard and Poisseau,³³ were able to show that the renal cells changed morphologically during salt and caffeine diuresis. In caffeine and in the diuresis produced by salts the epithelium decreased in size and showed greater prominence of its brush border. When hypotonic solutions were employed the epithelium increased in size.

Sollmann³⁴ and Schmitter³⁵ have both shown that sections of kidney tissue shrink in concentrated and swell in dilute salt solutions, the changes

in volume being due to an extraction or imbibition of fluid, which depends upon the strength of salt solution employed.

Williams,³⁶ working in Sollmann's laboratory, studied the cytological changes in the renal epithelium when subjected to the action of water, caffeine and various salts in different strength solutions. He employed both fresh and hardened sections. The histological study of the sections treated with the various solutions was not sufficient to explain the changes in weight and in gross appearance.

V. Sobieranski,³⁷ in a continuation of his work previously referred to, demonstrated histologically that the cells of the convoluted tubules were shrunk and the lumen widened in the kidneys of rabbits excised during diuresis from hypertonic salt solution. On the other hand, when .65 per cent hypotonic solution was employed the cells were swollen to an occlusion of the tubular lumen.

More recently Policard,³⁸ in studying the effect of salt solutions of various strengths on the cells of the convoluted tubules of white rats, has been able to show that the cells are altered in size by any change in the strength of the salt solution.

The foregoing review of the literature on experimental acute nephritis, though purposely limited, and to some extent selected for its bearing upon the epithelial pathology of the kidney, is sufficiently extensive to show the scope of previous investigations and to demonstrate in the majority of such investigations that the vascular reaction of the kidney has received the most attention, both anatomically and physiologically.

This investigation is principally concerned with the epithelial pathology of the kidney and is especially concerned with the part such changes may play in determining the quantitative output of urine.

Source of material: kidney poisons; dosage. — The animal employed in this study has been, without exception, the dog. No attempt has been made to use animals of even approximately the same age, though the use of animals either extremely old or young has been avoided, in the realization that extremes of age in either direction might modify a usually fairly constant reaction on the part of the kidney tissues.

Again, no attempt has been made to employ animals of approximately the same weight, for if the factor of age is

excluded in determining the weight, the tissue response to the various poisons should be fairly uniform, provided the quantity of poison employed is determined by the weight of the animal.

The experiments which form the basis of this work were commenced in 1908. The number of animals employed, though not perhaps excessive, is sufficient numerically and the constancy of results obtained will withstand conservative deductions.

Nephrotoxic substances which differed widely in their toxic effect have been employed for the purpose of observing the output of urine when either the vascular or the epithelial element of the kidney was the most affected. Finally, a poison has been employed which in appropriate doses with a suitable time limit for its action affects both elements of the kidney. By the use of a variety of poisons and by allowing rather wide latitude, so far as dosage was concerned, it has been possible to produce nephritides in which the vascular or epithelial pathology was dominant or in which both of these elements were fairly uniformly involved.

In such types of nephritis the output of urine has been studied in an attempt to determine which element of the kidney functioning pathologically has most influence in regulating the total output of urine.

The following is a list of the nephrotoxic substances employed, with their minimum and maximum dose:

Cantharidin: 1-10 milligrams per kilogram intravenously.

Cantharidin: 1 milligram per kilogram subcutaneously.

Potassium dichromate: 2.5-50 milligrams per kilogram subcutaneously.

Potassium dichromate: 5 milligrams per kilogram intravenously.

Sodium arsenate: 1-20 milligrams subcutaneously.

Bichloride of mercury: 10 cubic centimeters per kilogram of a 1-1000 solution.

Alcin: 2 cubic centimeters per kilogram of a 2 per cent solution.

Uranium nitrate: 2.5-15 milligrams subcutaneously.

In the cantharidin nephritis eight animals were used; in the potassium dichromate, nine; in the sodium arsenate,

four; in the uranium nitrate, six; and one each in the aloin and bichloride nephritis.

Technical methods employed. — The technic which has been employed in the experiments has been varied purposely in order to study the pathology of the kidney under slightly different conditions, though reacting to the same poison.

In the early experiments a technic was employed which would allow of both anatomical and physiological deductions. It was in these early experiments that it was noticed that the epithelial pathology showed marked variations, and that in those animals with a condition approaching an anuria there was fairly constantly associated with this state marked swelling of the renal epithelium.

The technical methods employed may be considered under the following headings:

1. Combined anatomical and physiological technic. — In this series of experiments (Series 5) under morphine-ether anesthesia manometric readings of the carotid pressure were made, the kidney poison was administered either intravenously, the femoral vein being used, or given subcutaneously. Alterations in the kidney volume were indicated by changes in the readings of a water manometer which was connected with a copper oncometer surrounding the left kidney.

The kidney flow was estimated by measuring the output in half-hour intervals from ureter canulas. In those experiments in which the oncometer was employed, no account was taken of the urine flow from the kidney in the oncometer, on account of the possibility of the flow being influenced by the pressure of the oncometer on the kidney or by its pressure upon the ureter and renal vessels.

The experiments were either allowed to come to a natural termination, after some hours of observation, or at different stages of the nephritis one kidney was removed and the tissue at once fixed to represent histologically one stage of the nephritis; and later in the experiment the remaining

kidney was removed to represent a more advanced stage of the same nephritis. By such a technic the anatomical alterations of the kidney at different stages of the nephritis could be studied with the total output of urine at these stages.

2. Anatomical technic. — The technic employed in the purely anatomical study was subjected to three modifications:

1. (Series 6.) Animals in a state of good nutrition and free from kidney involvement as indicated by the urine examination were kept in cages for three days, during which time the quantity of water consumed was not restricted. At the end of this time the animals were anesthetized with morphine-ether and with one exception the left kidney was removed as a control. The output of urine from the right kidney was studied while it was being subjected to the action of some nephrotoxic substance. The poison was administered either subcutaneously or intravenously.

In the animal above referred to which furnished the exception to the technic, the left kidney was not removed, but the flow of urine from both kidneys was determined while the animal was under the influence of the kidney poison. The object of not removing one kidney was to eliminate any effect such an operative procedure might have on the functional capacity of the intact kidney.

The urine flow in these animals was determined by measuring the output from a ureter canula in a constant unit of time.

2. The second modification of the anatomical technic consisted in selecting healthy animals, allowing them to remain in cages for three days, excluding a naturally acquired nephritis, and at the end of this time, under morphine-ether anesthesia in removing the left kidney as a control, allowing the animals to recover, and on subsequent days administering the kidney poisons subcutaneously.

With such a technic it was possible, in the first place, to obtain from an animal in which there was to be produced a

nephritis a normal kidney that could be used in a comparative study with the pathological kidney. Second, by terminating the nephritis artificially, or by allowing it to come to a natural termination, different stages of the pathological reaction on the part of the intact organ could be observed and compared with the normal, and observations could be made with special reference to the epithelial changes at these stages and to the associated output of urine.

3. In the third series of purely anatomical experiments the animals were kept in metabolism cages, a constant quantity of water was given the animals daily by the stomach tube and the urine was collected and studied qualitatively and microscopically.

After the existence of a nephritis was excluded and after a fairly accurate knowledge was obtained of the total output of urine to be expected from an animal receiving a definite quantity of water, the animals were given different nephrotoxic substances subcutaneously.

At different stages of the nephritis the animals were rapidly chloroformed, notes made of the gross pathology of the kidney, and kidney tissue fixed at once for microscopic study.

In this series of experiments, with the animals receiving daily a known amount of water, observations were made to determine if any correlation existed between the epithelial changes of the kidney at a given stage of the nephritis with the total output of urine.

In the majority of these experiments uranium nitrate was the kidney poison employed, since it produces a mixed nephritis, both vascular and tubular.

The relation which exists in the different types of nephritis between the epithelial changes and the total output of urine. — In the following discussion the experiments which constitute each series will be first considered separately and following the individual consideration the facts established by the series will be noted. Such a presentation has the disadvantage of materially increasing the length of this study, yet

it has the very obvious advantage of allowing the experiment to stand as an interpretation in itself.

In the description of the pathological anatomy of the kidney the epithelial changes are easily described and the general relation of the epithelium to the tubular lumen can be demonstrated by photomicrographs. On the other hand, the microscopic interpretation of the vascular pathology is more difficult, for the cytological changes are not nearly so clearly defined as are the epithelial changes. As a result of this structural difference in the elements of the kidney, terms which lack conciseness, and which in the description of the vascular reaction may fail to interpret the changes, must necessarily be employed, whereas it is possible to describe with some definiteness the epithelial reaction. For this reason it is safer to rely upon a physiological expression of the functional capacity of the vascular mechanism of the kidney than it is to attempt to determine the functional capacity of the vascular side of the kidney by histological alterations. However true this may be, it is also true that the vascular pathology can be interpreted histologically within certain limitations, especially when the vascular changes in a normal kidney are compared with those in a nephritic kidney.

The material which was employed for the microscopic study was obtained either during the experiment or very soon after death, so as to exclude the possibility of post-mortem changes.

The tissue was fixed in both corrosive-acetic and in formaline. In each experiment sections of uniform thickness were cut. The stains constantly used were hematoxylin and eosin. In staining for fat Soudan III. was employed.

PROTOCOLS.

Combined anatomical and physiological technic.

Series 5. Experiment No. 1.

Mongrel. Weight, 12 kilograms. Morphine-ether. Cantharidin, 5 milligrams per kilogram intravenously. Total cantharidin, 120 milligrams. At the time of the operation the animal's bladder contained 80 cubic centimeters of normal urine. Heart rate at the beginning of the experiment 136. Following the introduction of the first cantharidin the rate increased

to 168 and remained constant for one hour. During the following two and one-half hours the rate gradually decreased and at the termination of the experiment was 79. There was no acceleration from the subsequent use of cantharidin after the first injection.

During the course of the experiment the blood pressure remained fairly constant. At the commencement of the experiment the carotid pressure was 160 millimeters; at the termination 165 millimeters. Respirations varied from 36 to 19 per minute.

The urine flow was recorded from ureter canulas at half-hour intervals. During the first half-hour prior to the use of cantharidin there was no flow from the right ureter. The left ureter flow was 4 cubic centimeters. Following the first injection of cantharidin and during the second half-hour period the flow from the right ureter was 2 cubic centimeters, and from the left 5 cubic centimeters; during the third half-hour period albumen and hyaline casts appeared in the urine. The quantity of albumen and the number of casts showed a progressive increase and at the same time the quantity of urine increased until the termination of the experiment, at which time the left ureter flow was 5.5 cubic centimeters and the right 8.2 cubic centimeters.

The animal was killed by rapid chloroform anesthesia. Duration of experiment four hours.

The kidneys in the gross are intensely congested and have a tense feel. On section the cortical tissue bulges and drips blood freely. The glomeruli are prominent. The medulla shows macroscopically more evidence of congestion than does the cortex. The microscopic examination shows the inter-tubular and larger vessels engorged with blood. The glomerular capillaries are engorged with blood and fairly constantly fill the space enclosed by Bowman's capsule. There is occasionally a slight intra-capsular exudate.

The epithelium shows an early cloudy swelling with little if any encroachment upon the lumen of the tubules. The tubules contain granular detritus and occasional casts.

The experiment illustrates an early nephritis with a marked vascular reaction and with little response on the part of the epithelium. There is a progressive increase in the output of urine.

Series 5. Experiment No. 2.

Mongrel. Weight, 8 kilograms. Morphine-ether. Cantharidin, 5 milligrams per kilogram intravenously. Total cantharidin, 166 milligrams. No urine in the bladder at commencement of experiment. Carotid pressure at commencement of experiment 130 millimeters; at termination 120 millimeters. Heart rate varied from 78 to 65. Respiration from 24 to 9 per minute. During the first half-hour period prior to the injection of

cantharidin the urine flow from the right ureter was 4 cubic centimeters, from the left 0. The urine collected was normal. During the second half-hour the flow increased to 4.5 cubic centimeters from the left and 6 cubic centimeters from the right ureter. Following the administration of cantharidin the flow gradually decreased until at the close of the experiment, which lasted four and one-half hours, the right ureter flow was 1.2 cubic centimeters and the left two drops. Albumen hyaline casts and a few red blood cells appeared in the urine during the first half-hour after the injection of cantharidin.

The gross pathology of the kidney is similar to that described in the previous experiments, with the exception that the cortex appears pale when contrasted with the congested medulla.

The microscopic vascular pathology shows no notable difference. The epithelium, especially that of the convoluted tubules and of the ascending limb of Henle, is greatly swollen and vacuolated. The swelling very frequently completely occludes the lumen of the tubules. The nuclei of the epithelium are either small and stain deeply, or, as is the case in those cells most altered, are large and vesicular.

The experiment shows an early nephritis with a pronounced vascular disturbance, and in this it is comparable to the preceding experiment. On the other hand, it differs from the first experiment, in that the epithelium is strikingly involved and the flow of urine greatly decreased.

Series 5. Experiment No. 3.

Hound. Weight, 8.2 kilograms. Morphine-ether. Cantharidin, 5 milligrams per kilogram intravenously. Total cantharidin, 100 milligrams. Carotid pressure at commencement of experiment 135 millimeters, at termination 120 millimeters. Heart rate varied from 130 to 140, respiration 23 to 24 per minute. Oncometer pressure varied 5.8 centimeters (water manometer) to 6.2 centimeters. The normal flow of urine from the right ureter for the first two half-hour periods prior to the use of cantharidin was 4 cubic centimeters. Following the cantharidin it rose to 11 cubic centimeters, and during the next two half-hour periods decreased to 6.2 and 4.5 cubic centimeters respectively. The urine during these periods contained albumen, erythrocytes, and hyaline casts. The animal was rapidly killed by chloroform. Duration of experiment four and one-half hours. The kidney in the gross is a deep red color and drips blood freely on section. Microscopically the vessels are well filled, the glomerular capillaries usually completely fill the capsular space. The epithelium shows no change. The cytoplasm and nuclei stain normally.

The experiment shows an early cantharidin nephritis and an initial increase in the flow of urine following the injection

of cantharidin. The experiment was purposely terminated when the flow had reached the normal. The kidney shows a moderate grade of congestion with no epithelial changes.

Series 5. Experiment No. 4.

Mongrel. Weight, 12 kilograms. Morphine-ether. Cantharidin, 5 milligrams and 1 milligram per kilogram. Total cantharidin, 84 milligrams. Carotid pressure at commencement of experiment 110 millimeters, at termination 130 millimeters. Heart rate at beginning of experiment 86 per minute; at termination 84. Respiration 24 at beginning of experiment; at termination 20. Urine flow for the first half-hour period: right ureter 1.5 cubic centimeters; left .5 cubic centimeter. Urine normal. Second half-hour: 2.7 and 2.5 cubic centimeters from the right and left ureters respectively. Following cantharidin 5 milligrams, the urine flow increased to 3.8 cubic centimeters, right ureter; 3.5 cubic centimeters, left ureter. Trace of albumen and a few hyaline casts. With the following two half-hour periods 1 milligram of cantharidin was injected intravenously and the output of urine steadily increased as follows: 4.5, 3.5, 7, 7, 10, and 10 cubic centimeters — casts, principally hyaline, increased in number. The reaction for albumen became more pronounced and erythrocytes were numerous. Duration of experiment three hours. Animal was rapidly killed by chloroform. The kidney shows an intense congestion. On section the cortex bulges outward, and drips blood freely. Malpighian bodies are very prominent. Microscopically the intertubular vessels as well as the glomerular capillaries give the impression of being stuffed with blood. The capsular space is completely filled. There is occasionally a slight intertubular exudate. The epithelium stains normally; the cells may be considered to be in the early stages of cloudy swelling. The lumen of the tubules is not materially encroached upon by the epithelium. The tubules contain granular detritus and casts.

The experiment shows an early nephritis with an intense vascular reaction, but with slight epithelial involvement. The output of urine is increased.

Series 5. Experiment No. 5.

Setter bitch. Weight, 10 kilograms. Morphine-ether. Cantharidin, 1-.5 milligram per kilogram. Total cantharidin, 35 milligrams. Normal blood pressure, 144 millimeters. Blood pressure at termination of experiment, 148 millimeters. Heart rate varied between 96 and 76 per minute. Respirations 16 throughout experiment. Ureter flow for first two half-hour periods before the administration of cantharidin: right, 2.5 cubic centimeters; left, 2.5 cubic centimeters; right, 4 cubic centimeters; left,

4.7 cubic centimeters. Urine normal. Following 1 milligram of cantharidin per kilogram intravenously the urine dropped to 1.2 cubic centimeters from the right ureter and from left 2.5 cubic centimeters. Trace of albumen, few casts, erythrocytes. Following the second cantharidin injection the flow increased to 2.5 and 6 cubic centimeters from the right and left ureters respectively. With an increase in urine there was a decided increase in the quantity of albumen and in the number of casts and red blood cells. Two other injections of the kidney poison were made. The flow of urine rapidly decreased to .5 cubic centimeter, right ureter; 1 cubic centimeter, left ureter, and during the terminal half-hour there were only two drops of urine. Animal rapidly chloroformed. Duration of experiment three hours. The kidneys are of a deep red color, drip blood freely on section. Malpighian bodies are prominent. The cortex is distinctly paler than the medulla. Microscopically the vascular pathology is identical with that of the previous experiment. The epithelium, however, shows a striking difference. The epithelium of the convoluted tubules is principally affected and in these tubules cloudy swelling is extreme. The lumen of the tubules is often occluded by an approximation of the free border of the cells. The cells appear granular, nuclei indistinct and often vesicular. The epithelium in many of the tubules is vacuolated. Casts and granular material are found in the tubules in which the lumen has not been occluded.

In this experiment the vascular pathology is identical with that in the diuretic animal. On the other hand, in this anuric animal the epithelium encroachment upon, and obstruction of, the tubular lumen is decidedly marked.

Series 5. Experiment No. 6.

Male setter. Weight, 15 kilograms. Morphine-ether. Cantharidin, 1-2 milligrams per kilogram. Total cantharidin, 165 milligrams. The carotid pressure varied between 135 millimeters at the beginning of the experiment and 145 millimeters at the termination of the experiment. Minimum pressure 135 millimeters. The heart rate varied between 84 and 80 and the respirations between 32 and 15 per minute. The ureter flow for the two half-hours prior to the use of the cantharidin was as follows: right, 2 cubic centimeters; left, 8 cubic centimeters; right, 9 cubic centimeters; left, 3 cubic centimeters (kink in left ureter). Urine normal. Following the intravenous injection of 1 milligram of cantharidin per kilogram, which was repeated in twenty minutes, the urine flow from the right ureter was 5 cubic centimeters; left, 8 cubic centimeters. With the next period the output of urine from the right ureter was 5 cubic centimeters; left, 12 cubic centimeters. Trace of albumen. Few casts. Right kidney removed. For the subsequent two half-hour periods the left ureter flow was as follows: 20, 26, 38, 36, 40, 40, 20, 18, 10, and

6.6 cubic centimeters. There was a gradual increase in the quantity of albumen and in the number of casts and erythrocytes. The casts were rarely granular. Animal rapidly killed with chloroform. Duration of experiment seven hours. The right kidney, which was removed at the commencement of the nephritis and with the initial increase in the output of urine, shows macroscopically and microscopically a medium grade of congestion. It does not drip blood freely. The cortex does not bulge and the Malpighian bodies, though visible, are not excessively prominent. The intertubular and the glomerular vessels are fairly severely distended. There is usually a slight unfilled space between the glomerular vessels and Bowman's capsule. The epithelium, excepting that of the convoluted tubules, appears normal. The epithelium of these tubules show an early cloudy swelling and occasional vacuolation. The left kidney, however, which remained intact until the termination of the experiment, shows an unusually severe vascular reaction. It is of a bright red color. Drips blood freely from its cut surface. The cortex rolls out on section and the Malpighian bodies are very prominent. The intertubular vascular congestion is pronounced and the glomerular vessels fairly uniformly fill the capsular space. The nuclei of these capillaries and of those in the capsule are unusually prominent. The epithelium of the tubules shows advanced cloudy swelling and vacuolation. The tubular lumen is encroached upon and in some instances occluded. The epithelium stains imperfectly and looks hazy. Numerous casts and amorphous material are found in the tubules.

The experiment serves a double purpose of demonstrating an early and a late cantharidin nephritis. The kidney removed in the early stage of the nephritis shows fairly severe vascular changes and very slight epithelial changes. The kidney removed in the late nephritis shows an increase in the vascular pathology and pronounced epithelial alterations. There is a reduction in the output of urine, which, judging from the preceding observations, is approaching an anuria.

Anatomical study (first modification). Series 6. Experiment No. 1.

Pointer. Weight, 16.75 kilograms. Morphine-ether. Potassium dichromate, 2.5-5 milligrams per kilogram subcutaneously. Total potassium dichromate, 289.097 milligrams. Bladder contained 150 cubic centimeters of normal urine. Left kidney removed. Right ureter flow in first half-hour following nephrectomy, 2 cubic centimeters of normal urine. In first half-hour following 2.5 milligrams of potassium dichromate subcutaneously, 2.3 cubic centimeters. Urine contained a trace of albumen and a few hyaline casts. In the following half-hour period the urine

flow from the right ureter was as follows: 6 and 15.2 cubic centimeters, albumen casts, principally hyaline and erythrocytes. The left kidney, which was removed before the introduction of the chromate, was normal. The right kidney showed an intense congestion, dripped blood freely and the Malpighian bodies were prominent. Microscopically the glomerular and the intertubular vessels give the impression of being stuffed with blood cells. The endothelial nuclei are very prominent. The epithelium shows no swelling and no encroachment upon the lumen of the tubules. On the other hand, if the cell volume has changed, it has been in the direction of a shrinkage. The cytoplasm is not vacuolated. The cytoplasm and the nuclei stain normally. Numerous casts in the tubules.

The experiment represents an early chromate nephritis. The vascular pathology is pronounced. The epithelial changes are negative. There is an increase in the output of urine.

Series 6. Experiment No. 2.

Terrier bitch. Weight, 5 kilograms. Morphine-ether. Potassium dichromate, 5 milligrams per kilogram subcutaneously. Total potassium dichromate, 100 milligrams. Bladder contained 3 cubic centimeters of normal urine. Left kidney removed. Right ureter flow in first half-hour following nephrectomy, and prior to the use of the chromate 6.1 cubic centimeters of urine, which was normal. Following the first injection of the chromate the ureter flow was reduced to 1.2 cubic centimeters. Contained albumen casts and erythrocytes. The flow for the following half-hour periods, until the termination of the experiment, was as follows: 2, 4, 2.6, 8, and 8 cubic centimeters. Albumen casts and red blood cells showed a decided increase over the first observation. The animal was accidentally killed with ether. Duration of the experiment, three hours and fifteen minutes. The left kidney, which was removed before the chromate, was normal. The right kidney showed a fairly severe engorgement. It drips blood freely and the Malpighian bodies are prominent. Microscopically the vascular pathology is similar to that noted in the previous experiment. The epithelium shows little or no changes. It is not swollen, the cytoplasm and the nuclei stain well.

Here again the vascular pathology is decidedly evident. The epithelial involvement is negative. There is an increase in the output of urine.

Series 6. Experiment No. 3.

Hound bitch. Weight, 7 kilograms. Morphine-ether. Potassium dichromate, 5 milligrams per kilogram. Total dichromate, 315 milligrams. Urine obtained from bladder normal (not measured). Left kidney

removed. Right ureter flow in the half-hour period prior to the chromate injection: 2.7 cubic centimeters normal. Following the first subcutaneous injection of 5 milligrams of the chromate the urine flow was reduced to 2 cubic centimeters. The urine contained a trace of albumen and a few hyaline casts. Following this period the flow, for the following half-hour periods until the close of the experiment, was as follows: 12.5, 19, 7, 8.2, 2, 15.4, 22, and 26 centimeters. There was a progressive increase in the amount of albumen and in the number of casts and erythrocytes. Granular casts were numerous after the first two and one-half hours of the experiment. At the point in the experiment where the right ureter flow was reduced from 19 to 2 cubic centimeters the chromate had been pushed; 140 milligrams being injected in a two-hour period. Animal rapidly chloroformed. Duration of the experiment five and one-half hours. The left kidney, which was removed prior to the chromate injection, was normal. There was usually a distinct space between the glomerular capillaries and Bowman's membrane. The right kidney, which was removed at the termination of the experiment, showed a most intense congestion, as evidenced by the free dripping of blood from the cut surface, the tendency of the cortex to roll outward and the prominence of the Malpighian bodies.

Histologically the vascular pathology is more striking than in any of the previous experiments. The glomerular capillaries are packed with blood and not only fill the capsular space but give to Malpighian body the appearance of being distended and increased in size. The epithelial and the endothelial nuclei of the Malpighian body are increased in size, are strikingly prominent, and frequently stain poorly. The intertubular vessels participate equally in the vascular reaction. There is occasionally a slight intertubular exudate, containing erythrocytes. The epithelium of the tubules shows an early cloudy swelling, it appears granular and the nuclei stain imperfectly. The lumen of the tubules is only occasionally encroached upon by the altered epithelium. Casts and granular detritus are found in the tubules.

The experiment illustrates an early chromate nephritis in which the vascular pathology is marked and predominates over the epithelial changes. The epithelium shows an early cloudy swelling and has not encroached excessively upon the lumen of the tubules. The urine flow is greatly increased.

Series 6. Experiment No. 4.

Pointer bitch. Weight, 5 kilograms. Potassium dichromate, 50 milligrams per kilogram subcutaneously. Total chromate, 500 milligrams. Urine normal. June 8, 1910, 50 milligrams per kilogram of chromate subcutaneously. At the end of twenty-four hours the animal had voided only 25 cubic centimeters of bloody urine, highly albuminous, containing

numerous erythrocytes and casts. June 9, chromate injection repeated. Animal found dead in cage at 9 P.M. No urine in bladder. The post-mortem showed an intense gastro-enteritis. Both kidneys show a fairly intense congestion which macroscopically is most marked at the cortico-medullary boundary zone and in the medulla. Comparatively the cortex is pale. Microscopically the vascular reaction is not excessive. The glomerular capillaries, though well filled with blood, do not uniformly fill the capsular space. There is no intertubular exudate. The epithelium, especially that of the convoluted tubules, is severely swollen and vacuolated. The cytoplasm appears granular, the nuclei are large and stain imperfectly.

The experiment illustrates the striking affinity which the chromates may have for the renal epithelium. In this experiment the epithelial changes very clearly predominate; the tendency to occlusion of the tubular lumen is well marked. There is an anuria.

Anatomical study (second modification). Series 6. Experiment No. 5.

Mongrel. Weight, 6 kilograms. Morphine-ether anesthesia. Potassium dichromate, 50 milligrams per kilogram. Total chromate, 350 milligrams. June 8, 1910, left kidney removed. Urine normal. Fifty milligrams per kilogram of chromate injected subcutaneously. June 9, 1910, animal found dead in cage. Fifty-four cubic centimeters highly albuminous urine in bladder. Urine contains hyaline and granular casts and a few erythrocytes. The post-mortem showed a severe gastro-enteritis. The left kidney removed prior to the use of the chromate was normal. The right kidney showed an intense congestion, especially of the medulla and at the cortico medullary conjunction. Microscopically the capillaries of the Malpighian bodies fill and distend the capsule and are packed with blood. The intertubular vessels show the same change. Slight intertubular exudate. The nuclei of the capillary endothelium and of the epithelium of Bowman's membrane are quite prominent. In general the epithelium of the cortex and especially that of the convoluted tubules is greatly swollen. Cloudy swelling and vacuolation. The epithelium encroaches upon but only rarely occludes the tubules.

The experiment again shows the early epithelial involvement in the chromate nephritis and, with the epithelial changes, a reduced output of urine, fifty-four cubic centimeters.

Series 6. Experiment No. 6.

Terrier bitch. Weight, 7.5 kilograms. Morphine-ether. Potassium dichromate, 30 milligrams per kilogram subcutaneously. Total dichromate, 240 kilograms. Urine prior to the experiment normal. No urine in

the bladder at the time of the operation. Left kidney removed June 11, 1910. Chromate 30 milligrams per kilogram given subcutaneously. June 11, 1910, animal drinks water freely. No urine. Chromate injection repeated. June 12, 1910, animal found dead in cage. No urine. The post-mortem shows a fairly severe gastro-enteritis. No urine in the bladder. Left kidney normal. Right kidney presents an intense congestion of the medulla and cortico-medullary boundary zone. The cortex is pale. The microscopic examination shows an intense vascular reaction similar to the previous chromate experiments. In addition to the vascular changes enumerated in previous experiments, the glomerular capillaries show a beginning endothelial disintegration. The epithelium in general is involved. That of the convoluted tubules shows extensive cloudy swelling and necrosis. The nuclei are shrunken or fragmented and stain intensely. The tubular lumen is usually obliterated by the swollen, necrotic, and desquamated epithelium. In this chromate nephritis both vascular and epithelial elements of the kidney are extensively involved.

Judging from previous experiments the epithelial alterations preceded the structural vascular changes. Complete anuria.

Series 6. Experiment No. 7.

Male hound. Weight, 9.5 kilograms. Morphine-ether. Potassium dichromate, 20 milligrams per kilogram subcutaneously. Total dichromate, 350 milligrams. Urine prior to experiment normal. June 13, 1910, left kidney removed. Twenty milligrams per kilogram of chromate given subcutaneously. June 14, 1910, no urine. Animal very drowsy. Chromate injection repeated. June 15, 1910, no urine; animal very drowsy — not responsive to calls and whistles. No injection. June 16, 1910, 9 A.M., animal found dead in cage. Body warm, rigor mortis not complete. Gastro-enteritis. Bladder contained 30 cubic centimeters of highly albuminous urine with a few erythrocytes and very numerous finely and coarsely granular casts. The left kidney is normal. The right kidney shows the usual degree of congestion, — most marked in the medulla and at the cortico-medullary boundary zone. The cortex is pale. Microscopically the Malpighian bodies and intertubular vessels are structurally well preserved. The glomerular capillaries fill and distend the capsule, the nuclei are prominent. The epithelium, especially that of the convoluted tubules, shows a striking necrosis. In many cells the nuclei have disappeared. The epithelium of the ascending limb of Henle's loop and of the collecting tubules is fairly well preserved. The lumen of the tubules is usually obliterated. The condition is approaching an anuria, 30 cubic centimeters of urine in three days and a half.

Series 6. Experiment No. 8.

Terrier bitch. Weight, 9.5 kilograms. Morphine-ether. Sodium arsenate, 10 milligrams per kilogram subcutaneously. Total sodium arsenate, 760 milligrams. September 12th, left kidney removed. Ten milligrams of potassium arsenate subcutaneously. Urine normal. September 13th, urine 230 cubic centimeters normal; drinks water freely; injected. September 14th, injected; no urine. September 15th, injected. Animal voided urine twice during the day, most of which was lost. The urine collected was normal. September 16th, injected, 318 cubic centimeters urine; normal. September 17th, injected; animal drinks water freely, 274 cubic centimeters of urine, few hyaline casts, and a trace of albumen. September 18th, no arsenic; small quantity of urine, which was lost. During the following three days the animal was injected each day. The animal became excessively drowsy and on the third day became restless. Death without convulsions. Animal anuric for three days. The left kidney removed before the use of arsenic is normal. The right kidney is a deep red color, feels very tense. On section there is a free dripping of blood, the cortex bulges, the Malpighian bodies are prominent. Microscopically the glomerular capillaries are either distended with blood filling the capsular space or there has developed an exudate into the Malpighian body which compresses the capillaries. The endothelial and the epithelial nuclei are quite prominent. The intertubular vessels show the same congestion. The most striking feature of the histological picture is the pronounced intertubular exudate which separates and compresses the tubules, and which obliterates their lumen without the obliteration being dependent upon an increase in the size of the epithelium. The epithelium shows a fair grade of cloudy swelling and early desquamation. The desquamation is seen in tubules where the epithelium has not degenerated sufficiently to become loosened from its basement membrane. The shelving-off of the epithelium is more likely due to the exudate.

The experiment shows the gradual development of an anuria in a nephritis of the vascular type. Associated with the gradually acquired anuric state there are direct (cloudy swelling) and indirect (exudative) changes which tend to a narrowing or obliteration of the lumen of the tubules.

Series 6. Experiment No. 9.

Mongrel. Weight, 24.5 kilograms. Morphine-ether. Bichloride of mercury, 10 cubic centimeters of a 1-1,000 solution subcutaneously. Total bichloride of mercury, 70 milligrams. Sept. 30, 1910, left kidney removed, animal injected subcutaneously with 10 cubic centimeters of bichloride solution. The urine before the injection was normal. The

animal was injected on the succeeding seven days. The animal practically refused water. The urine flow varied between 60 and 180 cubic centimeters — daily — albumen and casts appeared on the fourth day. On the seventh day the animal was chloroformed. Extensive abscess from injections. The bladder contained 160 cubic centimeters of highly albuminous urine, which showed an occasional cast and a few erythrocytes. The left kidney was removed. The right kidney presented a fairly severe diffuse congestion. The Malpighian bodies were prominent. The microscopic examination confirmed the gross pathology. The epithelium showed a moderate degree of cloudy swelling.

The experiment represented an early nephritis from a heavy metal, slight epithelial involvement and an output of urine, which although diminished is not reduced to such a degree as to be considered approaching an anuria.

Series 6. Experiment No. 10.

Terrier bitch. Weight, 7 kilograms. Morphine-ether. Aloin, 2 cubic centimeters per kilogram of a 5 per cent solution subcutaneously. Total aloin, 14 cubic centimeters of a 5 per cent solution. Urine prior to aloin normal. The aloin injections were made on seven successive days. The output of urine varied but little. It remained normal until the seventh day, when a trace of albumen appeared. No casts, animal chloroformed on account of excessive abscesses. Both kidneys normal.

Series 6. Experiment No. 11.

Hound bitch. Weight, 7.5 kilograms. Cantharidin, 2.5 milligrams per kilogram. Total cantharidin, 14.25 milligrams subcutaneously. October 7th, urine normal; 20 cubic centimeters obtained from bladder. Left kidney removed. Cantharidin, 2.5 milligrams per kilogram subcutaneously. Animal died in convulsions at 10.30 A.M., on the following day. No urine in bladder. Left kidney removed. Right kidney fairly severely congested, which is most marked at cortico-medullary boundary zone. Cortex pale as compared with the medulla. Microscopically the vascular pathology, so far as the glomeruli are concerned, is not severe. The intertubular vessels are well choked with blood. The epithelium shows a surprising degree of cloudy swelling and desquamation without vacuolation. The lumen of the tubules is nearly uniformly closed. Casts are fairly numerous.

The experiment shows the very early involvement of the epithelium by a nephrotoxic substance, usually considered to be selectively vascular in its action, and with the early epithelial changes the rapid development of an anuria.

Series 6. Experiment No. 12.

Mongrel. Weight, 5.5 kilograms. Morphine-ether. Sodium arsenate, 10 milligrams per kilogram subcutaneously. Total sodium arsenate, 385 milligrams. Oct. 10, 1910, urine normal. Left kidney removed. Arsenic, 10 milligrams subcutaneously, no urine. Animal reinjected on the following day. No urine. During the following five days the animal received each day 10 milligrams of arsenic subcutaneously. The urine flow varied between 175-210 cubic centimeters. Albumen and a few hyaline casts and erythrocytes first appeared on the fourth day after the first injection. The animal was killed rapidly with chloroform on the eighth day of the experiment. Bladder contained 44 cubic centimeters of albuminous urine with numerous erythrocytes and a few hyaline casts. The left kidney was normal. The right kidney shows a most intense congestion which is only equaled by the kidney of the arsenical nephritis, previously described. The kidney feels very tense, is of a deep red color, on section the cortex turns outward and drips blood freely. The Malpighian bodies are very prominent. The microscopic study confirms the gross pathology as far as the vascular reaction is concerned. The glomerular capillaries are choked with blood and not only fill the capsular space, but have the appearance of distending it. The intertubular vessels participate equally in the congestion. There is no intertubular exudate. The epithelium of the convoluted tubules shows a slight cloudy swelling. The nuclei are indistinct; otherwise the epithelium is normal. Casts are occasionally seen. The animal was purposely chloroformed in the early stage of the arsenical nephritis.

The experiment demonstrates a most intense vascular nephritis, the epithelium is but little involved, the lumen of the tubules is not encroached upon either by swollen cells or by a compression of tubules by intertubular exudate. The output of urine is apparently but little interfered with. The experiment in all of its details should be compared with Experiment No. 8.

Series 6. Experiment No. 13.

Pointer. Weight, 9.3 kilograms. One milligram of cantharidin subcutaneously. Total cantharidin, 28 milligrams. Urine obtained prior to the experiment was normal. Oct. 14, 1910, left kidney removed. One milligram of cantharidin per kilogram subcutaneously. On the 15th the injection was repeated. A quantity of urine was lost, 30 cubic centimeters was recovered. This urine gave a pronounced test for albumen and contained numerous hyaline casts, a few granular casts and numerous erythrocytes. The animal received the third injection of the cantharidin on the following day. Urine 210 cubic centimeters, very albuminous, few hyaline

casts and numerous erythrocytes. The animal was chloroformed on the fourth day of the experiment. The bladder contained 54 cubic centimeters of urine which was highly albuminous and showed numerous casts and erythrocytes. The left kidney is normal. The right kidney shows a fairly diffuse congestion with prominent Malpighian bodies. The microscopic pathology demonstrates vascular changes of fair severity. No intertubular exudate. The epithelium shows little, if any, abnormality. Casts and granular detritus are found in the tubules. The output of urine is likely altered but little.

The experiment should be contrasted with Experiment No. 11 of this series. The vascular pathology in the two experiments is strikingly similar. The epithelial pathology is widely different. In Experiment No. 11 with marked epithelial changes an anuria developed quite rapidly. In Experiment No. 13, with an equally severe vascular disturbance and with only slight epithelial damage, the output of urine is uninfluenced.

Anatomical study (third modification). Series 10. Experiment No. 1.

Hound bitch. Weight, 3.85 kilograms. Potassium dichromate, 20 milligrams per kilogram subcutaneously. Total dichromate, 60.85 milligrams. Animal was given 200 cubic centimeters of water by stomach tube daily throughout the experiment. Dry diet. The urine flow on these days was as follows: 180, 180, 218 cubic centimeters. Normal. On the fourth day 20 milligrams per kilogram of chromate was given subcutaneously. On the day of injection the output of urine was 186 cubic centimeters. Normal. On the following day the urine increased to 278 cubic centimeters, was highly albuminous and contained numerous hyaline and granular casts and erythrocytes. Animal chloroformed. The kidneys in the gross and microscopically show an intense congestion. The endothelial nuclei are very prominent. The epithelium shows cloudy swelling and vacuolation and slight encroachment upon the lumen of the tubules. Convolted tubules are principally affected. Casts are numerous.

In this experiment there is an early diffuse nephritis, involving both the vascular and epithelial elements of the kidney. The epithelium has suffered least. The output of urine is increased.

Series 10. Experiment No. 2.

Hound bitch. Weight, 2.60 kilograms. Potassium dichromate, 10-20 milligrams per kilogram subcutaneously. Total dichromate, 78 milligrams. The animal was given 200 cubic centimeters of water by stomach tube

each day of the experiment. On the two days prior to the use of the chromate the urine flow was 181 and 163 cubic centimeters. On the third day 20 milligrams of chromate per kilogram was given subcutaneously. The urine flow was 340 cubic centimeters. Normal. On the following day 10 milligrams per kilogram of chromate was given. Urine flow 218 cubic centimeters. Highly albuminous, with numerous casts and erythrocytes. During the following day the urine flow was 88 cubic centimeters. The amount of albumen casts and erythrocytes greatly increased. Animal chloroformed. The kidneys, so far as the vascular reaction is concerned, are similarly affected as in the preceding experiment. The epithelium is greatly swollen and in areas necrotic. The epithelium of the collecting tubules and of Henle's loop is strikingly exempt from such changes.

The experiment shows a vascular reaction histologically comparable to that of the preceding experiment. It differs from the preceding experiment in the degree of epithelial involvement. The output of urine is reduced.

Series 10. Experiment No. 3.

Mongrel bitch. Weight, 2.18 kilograms. Potassium dichromate, 10-20 milligrams per kilogram subcutaneously. Total dichromate, 109 milligrams. Animal received 200 cubic centimeters of water by stomach tube daily. On the three days prior to the chromate the urine flow was 260, 275, and 264 cubic centimeters. Normal. On the fourth day the animal received 20 milligrams of chromate per kilogram, and on the three succeeding days 10 milligrams per kilogram. Urine flow, 190, 132, and 70 cubic centimeters. Terminal day, 25 cubic centimeters. Albumen casts and erythrocytes appeared in the urine within twelve hours after the first injection and increased in quantity each day. A reaction for sugar developed in the urine on the last day of the experiment. Animal chloroformed. The post-mortem shows a gastro-enteritis and a marked perirenal edema. The kidneys show a vascular reaction of fair severity. There are no histological vascular alterations, other than the prominence of the nuclei of Bowman's membrane and of the capillaries. The convoluted tubules are the principal seat of the epithelial changes, which consist in advanced cloudy swelling, necrosis, and desquamation, with obliteration of the lumen of the tubules. Casts are numerous. A condition approaching an anuria has developed.

Series 11. Experiment No. 1.

Hound bitch. Weight, 2.45 kilograms. Cantharidin, 1 milligram per kilogram. Total cantharidin, 9.8 milligrams. Animal received 150 cubic centimeters of water by stomach tube throughout the experiment. The urine flow on the days preceding the use of cantharidin was 174 and 188

cubic centimeters, and on the day of the first injection 170 cubic centimeters. Urine normal. Cantharidin, 1 milligram per kilogram was given subcutaneously on each succeeding day, except the last day of this experiment. The urine flow was as follows: 243, 225, 193, and 273 cubic centimeters. A trace of albumen appeared on the day following the first injection. The amount of albumen increased daily. A few casts and erythrocytes were present in the urine the last two days of the experiment. Animal chloroformed. The kidneys show a moderate grade of congestion. The Malpighian bodies are not generally well filled by the capillaries. The epithelial and endothelial nuclei are normal. The tubular epithelium appears shrunken, the tubular lumen is unusually large. The cytoplasm and the nuclei stain normally. An occasional cast is seen.

The experiment shows an early cantharidin nephritis, with a fairly severe vascular reaction, no epithelial reaction, and an increase in the output of urine.

Series 11. Experiment No. 2.

Hound bitch. Weight, 2.1 kilograms. Cantharidin, 1 milligram per kilogram subcutaneously. Total cantharidin, 2.1 milligrams. Animal received 150 cubic centimeters of water by stomach tube. Total daily output of urine before the kidney poison, 213 and 218 cubic centimeters. Normal. Following one injection of cantharidin the urine decreased to 161 cubic centimeters, was highly albuminous, and contained numerous hyaline and granular casts and erythrocytes. Chloroformed. The kidneys show a fairly severe congestion, the Malpighian bodies are very prominent, and well filled by the capillaries. The endothelium is not affected. The tubular epithelium shows an early cloudy swelling, vacuolation and encroachment upon the lumen of the tubules. The output of urine is decreasing.

Series 8. Experiment No. 1.

Hound bitch. Weight, 4 kilograms. Uranium nitrate, 10 milligrams. Total uranium, 30 milligrams subcutaneously. Animal received 200 cubic centimeters of water by stomach tube. Urine prior to uranium 170 and 165 cubic centimeters. Normal. One hundred and eighty-five cubic centimeters of normal urine on the first day of the injection. Injections were made on the next two days. On the first day the output of urine was 225 cubic centimeters, no sugar, pronounced reaction for albumen. Hyaline casts. The third day of the injections the urine decreased to 69 cubic centimeters, no sugar, albumen greatly increased. Very numerous hyaline and granular casts and erythrocytes. Chloroformed. The post-mortem failed to show either general or perinephric edema. The kidneys were severely congested, deep red in color and dripped blood

freely. The Malpighian bodies were prominent and contrasted strikingly with the pale cortex. The microscopic examination confirmed the gross pathology. The epithelium of the tubules shows cloudy swelling and an early necrosis. Rather frequently the epithelium, without much swelling, is shelved from its basement membrane into the tubular lumen. The lumen is partly or completely obstructed by the epithelial changes. The output of urine is decreased.

Series 8. Experiment No. 2.

Mongrel. Weight, 3.45 kilograms. Uranium nitrate, 10 milligrams daily. Total uranium, 60 milligrams. Two hundred and fifty cubic centimeters of water by stomach tube. Urine on the days prior to uranium, 175, 170, and 350 cubic centimeters. Normal. Except in the urine of the last day sugar was present. Ten milligrams of uranium was given subcutaneously for the succeeding days of the experiment. Urine 250, 190, 145, and 50 cubic centimeters, mostly vomitus, 10 cubic centimeters. Anuria for eleven hours. Albumen casts and erythrocytes appeared the day following the first injection. The glycosuria persisted. The post-mortem showed an edema of the subcutaneous tissue of the abdomen. The kidney showed a marked congestion, the Malpighian bodies were very prominent. The vascular changes were similar to the preceding experiment. The epithelium was in various stages of cloudy swelling, necrosis and desquamation. The disproportion between the histological alteration of the vascular and the epithelial elements of the kidney was striking. Anuria.

Series 8. Experiment No. 3.

Mongrel bitch. Weight, 4.2 kilograms. Uranium nitrate, 10 milligrams. Total uranium, 10 milligrams subcutaneously. Water, 250 cubic centimeters by stomach tube. Urine on the three days prior to uranium, 207, 350, and 330 cubic centimeters. Normal. Following 10 milligrams of uranium the animal developed a glycosuria and the urine increased to 457 cubic centimeters and contained albumen, hyaline and granular casts and erythrocytes. Animal chloroformed. No edema. The kidneys show an intense vascular reaction, and histologically the glomeruli are packed with blood and fairly uniformly fill the capsular space. The endothelial nuclei are very prominent. Occasionally the capillaries contain vacuoles. The epithelium is not swollen, but on the contrary appears to have undergone a shrinkage. The cytoplasm has a granular appearance and does not stain uniformly. The lumen of the tubules is not noticeably large. Occasional casts.

The experiment represents an early uranium nephritis in which the pathological changes are largely confined to the vascular element of the kidney. The epithelium has not

been seriously affected. The lumen of the tubules is not encroached upon. The output of urine has increased.

Series 8. Experiment No. 4.

Mongrel. Weight, 4.73 kilograms. Uranium nitrate, 15 milligrams subcutaneously. Total uranium, 30 milligrams. For four days the animal received 250 cubic centimeters of water by stomach tube and during this time the daily output of urine was 190, 200, 277, and 240 cubic centimeters. The water was increased to 300 cubic centimeters and the daily output of urine following the increase was 225, 400, 420, 450, 355, and 350 cubic centimeters. Urine normal. Fifteen milligrams of uranium were given and repeated the following day. The urine flow decreased as follows: 340, 100, 50, 125, and 160 cubic centimeters. The last two measurements contained a considerable quantity of vomitus. Albumen casts and erythrocytes appeared in the urine during the first twelve hours following the first injection and persisted. No sugar. The post-mortem shows no edema. The kidneys show the usual severe vascular reaction. The Malpighian bodies are well preserved. The epithelium shows extensive cloudy swelling, vacuolation, and necrosis. The nuclei have largely disappeared. Casts are quite numerous. The lumen of the tubules is encroached upon or obliterated by the epithelial changes.

The experiment illustrates a late uranium nephritis in which the vascular element of the kidney is singularly well preserved, while the epithelium has degenerated. There is likely an anuria. This cannot be accurately determined on account of the urine being mixed with vomitus.

Series 8. Experiment No. 5.

Hound. Weight, 2.5 kilograms. Uranium nitrate, 5 milligrams. Total uranium, 5 milligrams subcutaneously. Animal received 200 cubic centimeters of water by stomach tube for four days, including the day on which the uranium was injected. The output of urine was as follows: 84, 70, 90, and 77 cubic centimeters. Urine normal. The day following the uranium injection the urine increased to 102 cubic centimeters and with this the animal developed a glycosuria. The urine gave a pronounced reaction for albumen and contained hyaline and granular casts and erythrocytes. Chloroformed. The kidneys show the usual congestion and microscopically the glomerular vessels are well filled with blood and fairly well fill the space enclosed by Bowman's membrane. The tubular epithelium shows a rather prominent brush border, stains well and appears shrunken. The lumen of the tubules is noticeably large. Occasional casts. The experiment shows a moderately severe vascular reaction. The epithelium is not involved to the extent of diminishing the tubular lumen. The urine is increased.

Series 8. Experiment No. 6.

Hound bitch. Weight, 2.68 kilograms. Uranium nitrate, .5-2.5 milligrams subcutaneously. Total uranium, 26.8 milligrams. Two hundred cubic centimeters of water daily by stomach tube. Urine for three days, including the amount noted at the time of the first injection of uranium, 241, 300, and 217 cubic centimeters. Normal. The day following the injection the urine increased to 350 cubic centimeters, the animal developed a glycosuria; albumen in abundance, few hyaline and granular casts and erythrocytes. The following two days the urine dropped to 120 and 15 cubic centimeters and contained vomitus, albumen, sugar, casts, and blood cells. Chloroformed. The post-mortem showed a marked subcutaneous and perirenal edema. The kidney showed an intense congestion. Microscopically the glomeruli fill the capsule, the nuclei are enlarged. Occasionally there is a fibrinous exudate into the Malpighian bodies and stroma of the kidney. The epithelium is necrotic, desquamated and obliterating the lumen of the tubules. The epithelium of Henle's loop tubules is fairly well preserved. Casts numerous. The contrast between the degree of preservation of the vascular element and the epithelium is marked. Anuria.

Series 6. Experiment No. 7.

Mongrel. Weight, 3 kilograms. Sodium arsenate, 1-10 milligrams. The animal has been under observation for sixty days. Each day the animal has received 160 cubic centimeters of water by stomach tube. For nine days prior to the commencement of the arsenate injection the output of urine was 240, 300, 285, 260, 280, 210, 235, 176, and 230 cubic centimeters daily. The urine was normal. For eight days the animal received 1.5 milligrams of sodium arsenate, for five days 5 milligrams, and since the fourteenth day to the present time 10 milligrams. Commencing with the arsenical injections the urine increased to 260 cubic centimeters and on the next day to 400 cubic centimeters; gave a moderate reaction for albumen, contained a few hyaline casts and erythrocytes. The output of urine has varied between a minimum of 175 cubic centimeters and a maximum of 500 cubic centimeters. The urine flow on the sixtieth day of the experiment was 260 cubic centimeters. The presence of albumen has been constant though distinctly variable in amount. On six days during the experiment no casts were found, on the remaining days they were present, though scant. The animal has increased in weight, now weighing 6.85 kilograms.

Series 6. Experiment No. 8.

Mongrel. Weight, 3.15 kilograms. Sodium arsenate, 3-10 milligrams. The technic employed in this experiment has been identical in every particular with that employed in the preceding experiment. The output of urine for the nine days before the use of the arsenic was 225, 265, 260,

210, 230, 200, 300, and 300 cubic centimeters daily. The urine was normal. Since the commencement of the arsenate injections the urine has varied between 170-640 cubic centimeters. The output of urine on the sixtieth day is 270 cubic centimeters. Albumen in variable quantities has been constantly present. Hyaline casts and erythrocytes have been demonstrated in the urine on thirty-eight of the sixty days. The present weight of the animal is 7.25 kilograms.

The last two experiments may be considered together. Judging from the urinalyses the animals have a nephritis. Judging from the other arsenical experiments enumerated in this paper and from the experimental work of others, these animals have had a vascular nephritis, likely of slight severity, but severe enough to show albumen, casts, and erythrocytes in the urine for thirty days. During this time the output of urine has not been reduced.

SUMMARY.

At the conclusion of each experiment contained in the protocols, a note has been made of the vascular reaction of the kidney, the extent to which the epithelium has been involved; and also the relation which exists between the degree of epithelial involvement and the total output of urine has been recorded.

The following summary combines these elements of the experiments for the different series:

Series No. 5. — Six animals were employed in this series and the observations made were both anatomical and physiological. Cantharidin, which was the nephrotoxic substance used, was given intravenously.

Four of the six animals showed a temporary increase in the output of urine following the initial injection of cantharidin. Observations similar to this, relative to the diuresis produced by cantharidin, have been previously referred to. In three of these nephritic and diuretic animals the experiment was terminated during this stage of the nephritis; while in the remaining animals of the series the kidney reaction was allowed to continue until either the output of urine had returned to approximately what it was at the commencement

of the experiment, or the output was greatly reduced, approaching a condition of anuria.

In none of the kidneys of those animals examined histologically before any reduction in the output of urine below the normal had developed, although the animal had an active nephritis as indicated by the urinalyses and by the pathology of the kidney, were the epithelial alterations of such a character as to encroach upon or materially influence the size of the lumen of the tubules. On the other hand, in those animals which showed prior to the termination of the experiment a reduction in the output of urine the epithelial changes were prominent in the pathology of the kidney and tended towards an obliteration of the tubular lumen. The difference in the degree of epithelial involvement is clearly seen by comparing Figs. 1 and 2 of Experiments 4 and 5.

In this series of experiments the vascular pathology, so far as it can be interpreted by histological alterations, does not show the same association between the severity of the changes and the output of urine, as is shown by the epithelium. For example, in Experiments 4 and 5 the vascular pathology is practically identical in the two experiments. The gross pathology of the kidneys gives every evidence of a severe vascular disturbance and the microscopic pathology confirms this, yet in Experiment 4 the output of urine is increased, while in Experiment 5 we get an anuria.

In this first series of experiments physiological observations were made before the purely anatomical study was decided upon. The results of these observations will therefore be incidentally incorporated in the discussion of this series.

Oncometric determinations of the kidney volume were made in only one experiment. These will not be considered.

The carotid blood pressure remained remarkably constant throughout the experiment. In those animals which either developed an anuria, or a condition approaching it, the fact that the general blood pressure remained as high as or higher than it was at the commencement of the experiment excludes a general circulatory failure as being a factor in decreasing

the output of urine. The elimination of this possible factor in the development of an anuria does not, of course, exclude the possibility of a local circulatory failure in the kidney.

The rate of the heart was temporarily increased in three of the experiments; and accompanying the increase in rate there was a temporary rise in arterial pressure. It is interesting to note that, associated with this change in the circulation, there develops the initial increase in the flow of urine.

In all of the animals, with one exception, there was a progressive decrease in the respiratory rate. In the excepted animal the respirations remained constant throughout the experiment.

Series No. 6. — The animals employed in this series furnished a purely anatomical study. The technic has undergone three modifications.

The first three animals of this series were allowed to remain in cages for the three days before the use of the nephrotoxic substance, which in each instance was potassium dichromate. During this period the intake of fluid was not restricted. The urine, which was examined daily, was normal.

The experiments consisted in anesthetizing the animals, removing one kidney as a control, introducing a ureter canula into the ureter of the intact kidney, and in observing the flow of urine at half-hour periods. The chromate was given subcutaneously.

The first animal received 289.97 milligrams of the chromate, the second one hundred, and the third three hundred and fifteen milligrams. All of the animals showed the presence of albumen and casts in the urine within half-hour of the initial injection of the chromate. The amount of albumen and the number of casts showed an increase until the termination of the experiment.

In Experiment No. 1 the output of urine increased from two cubic centimeters per half-hour before the injection to fifteen and two-tenths cubic centimeters, at which time the intact kidney was removed and the animal killed.

In the third experiment the same increase in the urine

from the chromate was observed. In this instance the urine flow varied from two and seven-tenths cubic centimeters before the chromate to twenty-six cubic centimeters at the close of the experiment.

The second experiment gave a similar increase, though to a less degree.

The effect of the chromate in these experiments on the flow of urine was far from what had been expected.

Judging from the work of others and assuming that the epithelial changes had an influence in determining the quantitative output of urine, it was with this kidney poison that an anuria was expected to develop rapidly, on account of the known tendency of the chromates to produce a tubular nephritis.

As will be seen from a study of the protocols, the pathology of the kidney in an early chromate nephritis bears a striking resemblance to the early nephritis from cantharidin.

In the three chromate nephritides just mentioned, the vascular element of the kidney is the element which is first affected and which shows the most notable change. The epithelial element in these early experiments is but slightly involved and the cells not only do not show an encroachment upon the lumen of the tubules but in Experiment No. 1, if any change in volume has taken place, it has been in the direction of a shrinkage (Figs. 3 and 4).

Judging from other chromate experiments and from experiments with other nephrotoxic substances, these polyuric animals with a chromate nephritis, if they had been given sufficient time, would have developed an anuria. Furthermore, the development of the anuria would probably have taken place much more rapidly on account of the undoubted affinity of the chromates for the tubular epithelium than would have been the case if poisons, such, for instance, as arsenic or cantharidin had been employed, which have more predilection for the vascular side of the kidney.

The fourth animal of the series was not subjected to the effect of a nephrectomy or to an anesthetic, but was given

the chromate in a much larger dose on two successive days. Prior to the injection the animal was voiding normal urine. After the first injection only twenty-five cubic centimeters of bloody, highly albuminous urine containing casts was voided. The animal died on the second day of the second injection.

The kidney showed a fairly severe vascular reaction and a pronounced reaction on the part of the tubular epithelium, which was greatly swollen and which encroached upon, or occluded the tubules. On the first day the output of urine was greatly reduced and on the second day the animal was anuric.

The kidneys of this animal represent a later stage, and the usually observed stage of a chromate nephritis, in which the tubular nephritis is more marked than the vascular (Figs. 5 and 6.)

The second modification of the technic employed in the anatomical study consisted in selecting animals in a state of good nutrition, excluding the presence of a naturally acquired nephritis, and, after three days of preliminary observation, in removing the left kidney as a control and then in injecting various kidney poisons subcutaneously.

The intake of fluid by these animals was not restricted and, as the quantity of fluid consumed by the different ones varied, the output of urine would also vary necessarily. The anatomical study of the kidney has, therefore, a relative rather than an absolute value in interpreting the influence of the pathology incident in the kidney upon the total output of urine.

In the next series of animals to be discussed, in which an accurate account was made of the intake of fluid by the animals and in which the same nephrotoxic substances were employed, the results obtained substantiate the observations made in the series now under consideration.

The first three animals used in this series were subjected to a chromate nephritis. The first animal (No. 5) received three hundred milligrams of the chromate; the second (No. 6), four hundred and fifty; and the third (No. 7), three

hundred and sixty. In all three, either a complete anuria or a condition approaching an anuria developed rapidly. In none was there a period in which the output of urine was increased.

The pathology of the kidney shows a severe vascular nephritis and accompanying these changes equally severe changes in the tubular epithelium, which has either led to a complete occlusion of the tubules or to a marked reduction in the tubular lumen.

These experiments should be contrasted with the diuretic experiments from the chromates which have been previously discussed. In these experiments smaller quantities of the chromate were administered and the experiments were terminated artificially at an early stage in the nephritis.

In the diuretic experiments, although the vascular pathology was pronounced, the epithelium was but little involved, and the output of urine was not only not reduced but had in fact undergone an increase.

In the experiments just described, which represent a later stage of the nephritis, characterized by epithelial involvement, the urine is either greatly reduced or an anuria has developed (Figs. 7, 8, and 9).

Two of the animals employed in this series were subjected to an arsenical nephritis.

The first (No. 8), after the removal of the left kidney, which later proved to be normal, was given subcutaneously ten milligrams per kilogram of the arsenic solution on six successive days; then with an intermission of one day the injections were resumed for the following two days.

As will be seen from the protocol, although large doses of the poison were being used and frequently repeated, the output of urine was certainly not greatly reduced even when the animal had developed an albuminuria with casts. On the second day following this observation the animal became anuric and this state persisted until its death, three days later.

The renal pathology in this animal is extremely interesting as it possibly furnishes an explanation for the delayed

anuria when large doses (ninety-five milligrams) of the poison were being administered daily.

The vascular pathology, which is especially striking and which has led to the formation of an unusually large inter-tubular and intraglomerular exudate, was probably gradual in its development. It was perhaps present to some extent when the urine flow was two hundred and forty cubic centimeters and contained albumen and casts. The anuria developed two days after this indication of vascular injury and it was probably during this period that enough exudate between the tubules had been formed either to greatly lessen their lumen or to cause it to become obliterated by compression of the tubules by the exudate. In this nephritis, although the epithelium is involved in the reaction and is to some extent increased in volume, the principal change which has reduced, or obliterated to a slit-like opening, the tubular lumen has been the compression of the tubules by the exudate.

The occasional compression of the glomerular capillaries by the intraglomerular exudate is another factor which has to be considered in this experiment as contributing to the development of the anuria.

It has been shown in investigations which have been previously referred to that arsenic produces more nearly an exclusively vascular nephritis than does any other nephrotoxic substance, and, also, that the output of urine is least affected by this poison.

These observations may find an explanation in the fact that arsenic has but slight initial affinity for the tubular epithelium and that an anuria from arsenic developed when the vascular pathology is of such severity as to involve indirectly the epithelial element of the kidney. In this connection there must also be considered the late effect of arsenic directly upon the epithelium, which increases its volume and contributes to the same results (Figs. 10, 11, and 12).

The second arsenical experiment (No. 12) in this series tends to substantiate this view.

This animal received fifty-five milligrams of arsenic on

seven successive days. The daily flow of urine varied between 175–210 cubic centimeters, and when the animal was chloroformed on the eighth day the bladder contained forty-four cubic centimeters of fairly albuminous urine, with numerous erythrocytes and a few casts.

Pathologically the kidney showed an intense vascular reaction which had, however, not reached the stage of exudate formation. The lumen of the tubules was not encroached upon either by changes in the epithelium or by compression of the tubules. The output of urine was evidently not greatly reduced (Fig. 13).

In two experiments which are now in progress (No. 7 and No. 8) the observations which have been made also tend to support the explanation, which has been ventured, for the development of an anuria in an arsenical nephritis.

The remaining experiments in this series consist of two with cantharidin and one each with aloin and bichloride of mercury. The last two will not be discussed as they furnished insufficient data.

The cantharidin animals (No. 11 and Np. 13) show an anuric type of nephritis, and also a type in which, with a marked vascular pathology, the output of urine is not greatly reduced.

In the former experiments the epithelial degeneration has developed surprisingly early and serves to show the extent to which this element of the kidney may be affected by cantharidin, a poison usually supposed to have such a selective action on the vessels.

The latter experiment shows the usual type of cantharidin nephritis in which the vascular pathology predominates over that of the epithelium (Figs. 14, 15, and 16).

The third modification of the anatomical technic which was employed in Series 10, 11, and 8 differed from the technic used in Series 6, in that a known quantity of water was given to the animals each day. With this modification a check would be had on the observations made relative to the total output of urine in the animals of Series 6, in which the intake of water was not restricted.

The kidney poisons used in these series of experiments were potassium dichromate to represent the epithelial or tubular type of nephritis, arsenic and cantharidin to represent the vascular type, and uranium nitrate to represent a combination of both types.

In Series 10 three animals were used for the chromate nephritis. The chromate was administered subcutaneously. The animals received two hundred cubic centimeters of water daily by stomach tube.

The first animal, before the chromate was used, had a daily flow of urine varying between one hundred and eighty and two hundred and eighteen cubic centimeters. The urine was normal. On the day of the first chromate injection the urine flow was one hundred and eighty-six cubic centimeters, on the following day it had increased to two hundred and seventy-eight cubic centimeters, was very rich in albumen and contained numerous hyaline and granular casts and erythrocytes. The animal was chloroformed in this stage of the chromate nephritis, in which the animal is polyuric.

The kidney showed a diffuse nephritis in which both the vascular and the epithelial elements of the kidney were involved. The vascular pathology was histologically more pronounced than was the epithelial pathology. Urine flow was not only not reduced but was increased.

The remaining experiments of this series were allowed to continue until the output of urine was either reduced or the animal was practically anuric.

Histologically the vascular pathology in these experiments is identical with the vascular pathology in the previous experiment. The epithelial degeneration is, however, much more extensive, and the lumen of the tubules is more uniformly encroached upon by the altered epithelium.

In Series 11 cantharidin was employed subcutaneously. The animals were given one hundred and fifty cubic centimeters of water daily.

The first animal of the series was chloroformed on the fourth day of the nephritis, when the urine had increased from one hundred and seventy cubic centimeters prior to the

cantharidin to two hundred and seventy-three cubic centimeters.

The kidneys showed the usual severe vascular nephritis that is produced by this poison. The epithelial involvement was slight.

The second experiment of this group was terminated when the output of urine had been reduced from two hundred and eighteen to one hundred and sixty-one cubic centimeters. The vascular pathology differed in no way from that of the preceding experiment. The epithelium showed a well defined swelling, vacuolation, and encroachment upon the tubular lumen.

It will be noted that in the experiments of Series 10 and 11, in which a known quantity of fluid was daily consumed, the results obtained substantiate the observations made in the animals in Series 6 in which the intake of fluid was not restricted.

Briefly stated, these observations are, that in animals with an acute nephritis there is a constant association between the severity of the epithelial changes and the total output of urine, and secondly, that the vascular reaction may be histologically as severe in a polyuric as it is in an anuric animal.

In the final series of animals employed in this study uranium nitrate was the nephrotoxic substance selected on account of its supposed ability to involve more nearly at the same time both the vascular and the epithelial elements of the kidney.

The six animals representing the series were subjected to the usual period of preliminary observations. The quantity of water which was daily allowed them varied with their weight. The smaller animals received two hundred cubic centimeters; the larger two hundred and fifty cubic centimeters. The uranium was given subcutaneously in doses varying from two and one-half to ten milligrams per animal.

At different periods during the nephritis which represented an increased output of urine, a decrease, or an anuria, the animals were rapidly chloroformed and the kidney tissue obtained for histological study.

The first experiment of the series shows an initial increase in the output of urine which is accompanied by the development of a well-marked albuminuria and the presence in the urine of casts. Following this period the urine was greatly reduced in amount. The quantity of albumen was increased, the casts were more numerous and erythrocytes were present in large numbers.

The kidneys show a nephritis in which the vascular changes predominate. The epithelium shows cloudy swelling and early necrosis, vacuolation, and occasional desquamation.

In this animal both elements of the kidney are involved. The vascular pathology though decidedly pronounced is no more extensive than in animals diuretic from other kidney poisons.

The epithelial changes, which are also pronounced, are similar to the changes which have been noted during the course of this investigation and which have been associated with either a reduction in the output of urine or with an anuria (Fig. 17).

The second animal of this series was subjected to the action of the poison for a longer period than was the first. Like the first, there developed in this one, following the initial injection of uranium, a period during which the output of urine was increased. This was followed by a gradual decrease which terminated in an anuria for eleven hours prior to the animal's death.

The kidneys show the most extreme grade of epithelial involvement, which is indicated by the necrosis of the cells of the convoluted tubules and by cloudy swelling and desquamation in different degrees of severity of the epithelium in general. The Malpighian bodies are remarkably well preserved, considering the severe changes in the tubules (Fig. 18).

Experiments 3 and 5 in this series, which were terminated during the period of increased urine flow, though showing a vascular pathology which histologically is comparable to that noted in the first experiment, present a striking contrast in the epithelial pathology.

In Experiment 3 there has been an increase in the urine flow over the normal of one hundred and twenty-seven cubic centimeters. The urine was albuminous and contained hyaline casts and granular casts and erythrocytes. The animal was glycosuric.

In Experiment 5 there was an increase in urine following the uranium of twenty-three cubic centimeters. The animal was glycosuric and had a urine rich in albumen. The urine contained erythrocytes, hyaline and granular casts.

In both of these experiments, in which there was every evidence of a pronounced vascular nephritis, the output of urine was increased.

The epithelial changes in the kidneys of these animals are remarkably slight. There is certainly no change which tends towards an occlusion of the tubules by the altered epithelium, but on the other hand the epithelium gives the appearance of having undergone a shrinkage (Figs. 19 and 20).

The association in these nephritic animals of a glycosuria, increased diuresis, and shrunken tubular epithelium is certainly of interest and has been considered in a physiological study of uranium nephritis.²⁹

The two remaining experiments in this series, Animals 4 and 6, represent a later stage in the nephritis, in which with a histologically intact vascular mechanism but with severe epithelial changes, the output of urine has been either greatly reduced or an anuria has developed (Fig. 21).

The relative affinity of the various poisons for the epithelial and for the vascular elements of the kidney. — The affinity of the different nephrotoxic substances which have been used in this investigation for the different tissues of kidneys agree in general with the classification made by Pearce.¹¹

Thus the chromates usually give a nephritis which is more tubular than glomerular, while arsenic certainly exerts its chief influence on the vascular element of the kidney. However true this may be, it has been shown in several

experiments which have been discussed in this study that provided an epithelial poison be given in a small enough quantity and provided the ensuing nephritis be terminated early, a nephritis can be obtained which is almost purely vascular. This point has been illustrated in animals with a typical epithelial poison such as potassium dichromate.

On the other hand, it has also been demonstrated that when a vascular poison is employed, such as cantharidin, a tubular nephritis can be obtained, provided a sufficient amount of the poison be used, or in case the nephrotoxic substance be used in small quantities, that the tubular nephritis develops, provided sufficient time be allowed for the development of this reaction on the part of the kidney.

Of the kidney poisons used in this investigation arsenic is undoubtedly the most selective in its action, producing a vascular nephritis with a very late involvement of the epithelium. Even when this involvement takes place it is, comparatively speaking, slight, and may arise secondarily to the vascular pathology and not be due to any direct action of the poison on the renal epithelium.

Lastly, with all of the nephrotoxic substances employed there is a stage early in the nephritis when the reaction by the kidney is almost purely vascular. If this reaction has been produced by a tubular poison the epithelium of the kidney will very early participate in the nephritis, while if the early vascular nephritis has been produced by a vascular poison the tubules will be affected late in the nephritis.

CONCLUSIONS.

From the experimental data presented in this investigation the following conclusions appear allowable:

(1.) Cantharidin, potassium dichromate, uranium nitrate, and sodium arsenate produce in the dog an acute nephritis in which both the vascular and the epithelial elements of the kidney are involved.

(2.) The histological study shows that the vascular element of the kidney is first affected and that the rapidity with which the epithelium is involved depends principally upon

the nephrotoxic substances employed in producing the nephritis.

(3.) Uranium nitrate and potassium dichromate usually produce a tubular nephritis much earlier than either cantharidin or sodium arsenate.

(4.) In the early stages of the nephritis from these poisons, when there is anatomical evidence of vascular injury and either slight or no anatomical evidence of epithelial injury, the output of urine is increased.

(5.) Later in the nephritis, when histologically the vascular pathology may not be increased in severity, but when the epithelium has become involved, the output of urine is reduced or an anuria is established.

(6.) Those nephrotoxic substances which have the most marked affinity for the tubular epithelium are the substances which most rapidly cause either a reduced output of urine or an anuria, while those nephrotoxic substances, such as arsenic, which cause an early and a pronounced vascular injury, with late epithelial involvement, are the poisons which have the least tendency to produce an anuria.

(7.) In the experiments detailed in this study which have shown either a pronounced decrease in the output of urine or an anuria, there has constantly been associated epithelial changes, which would produce in different degrees an obstruction of the lumen of the tubules.

(8.) In those experiments in which the output of urine has not been decreased, and in those experiments in which the output of urine was increased beyond the normal, such epithelial changes either did not exist or they were histologically slight.

(9.) Finally, the investigation does not presume to interpret, except in a most general way, the physiological reaction of a nephritic kidney. This aspect of the problem will be studied with an appropriate physiological technic.

[I wish to express my appreciation to Dr. Torald Sollmann for his kindness in going over with me the microscopic preparations and for reviewing the experimental data.]

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DESCRIPTION OF PLATES.

PLATE III.

FIG. 1. — B. and L. obj. 3, oc. 1. Series 5, No. 4. The figure shows the kidney in an early cantharidin nephritis with an increase in the output of urine. The glomerular capillaries completely fill the space enclosed by Bowman's membrane, the nuclei of the capillaries and of the capsule are prominent. The tubular epithelium is but little involved. A cast is seen in one of the tubules.

FIG. 2. — B. and L. obj. 3, oc. 1. Series 5, No. 5. The figure shows the kidney in a late cantharidin nephritis with an output of only a few

drops of urine in a half-hour period. The glomerular vessels are packed with blood and fairly uniformly fill the capsule. The epithelium shows a marked grade of swelling and obliteration of the tubular lumen.

FIG. 3. — B. and L. obj. 3, oc. 1. Series 6, No. 1. The figure represents a chromate nephritis in the stage of polyuria. The glomerulus is distended by the engorged capillaries. The epithelium shows no swelling. The tubules contain granular detritus.

FIG. 4. — B. and L. obj. 3, oc. 1. Series 6, No. 2. This figure also represents the kidney in an early chromate nephritis. The epithelium has not encroached upon the tubular lumen. The tubules contain granular detritus.

PLATE IV.

FIGS. 5 and 6. — B. and L. obj. 3, oc. 1. Series 6, No. 4. The figures represent the kidney in a later stage of chromate nephritis in which an anuria had developed. The swelling and occlusion of the lumen of the tubules by the epithelium is far more marked than is the vascular pathology.

FIG. 7. — B. and L. obj. 3, oc. 1. Series 6, No. 6. The figure shows a very late stage in the chromate nephritis. The epithelium has undergone extensive necrosis and the outline of the tubules is no longer preserved. The glomeruli, which show a marked degeneration, are fairly well preserved, considering the extent of the epithelial destruction.

FIG. 8. — B. and L. obj. 3, oc. 1. Series 6, No. 7. The figure represents the kidney before the use of the chromate. The glomerular capillaries do not fill the space enclosed by Bowman's membrane. The epithelium shows no encroachment upon the lumen of the tubules.

FIG. 9. — B. and L. obj. 3, oc. 1. Series 6, No. 7. The figure shows the kidney of the same animal in a late chromate nephritis, with anuria. The glomerular vessels have filled the space enclosed by the capsule and show an early disintegration. The epithelium is necrotic. The epithelial changes are more extensive than the vascular.

FIG. 10. — B. and L. obj. 3, oc. 1. Series 6, No. 8. The figure represents the kidney of animal No. 8 before the use of a nephrotoxic substance.

PLATE V.

FIGS. 11 and 12. — B. and L. obj. 3, oc. 1. Series 6, No. 8. These figures represent the kidney of the same animal, anuric from arsenic. The vascular pathology is especially marked. The Malpighian bodies show an extensive intra-capsular exudate with compression of the glomerular capillaries. There is a marked intertubular exudate. The tubules are frequently occluded. The occlusion has been caused more by the compression of the exudate than by changes in the epithelium.

FIG. 13. — B. and L. obj. 3, oc. 1. Series 6, No. 12. The figure represents the kidney in an arsenic nephritis without a reduction in the output of urine. The vascular pathology is marked. The tubular involvement is slight. No exudate has been formed to compress the tubules.

FIG. 14. — B. and L. obj. 3, oc. 1. Series 6, No. 11. The figure represents the kidney of an animal that developed an early anuria from cantharidin. There is an extensive necrosis of the tubular epithelium.

FIG. 15. — B. and L. obj. 3, oc. 1. Series 6, No. 13. The figure represents the kidney before the use of cantharidin.

PLATE VI.

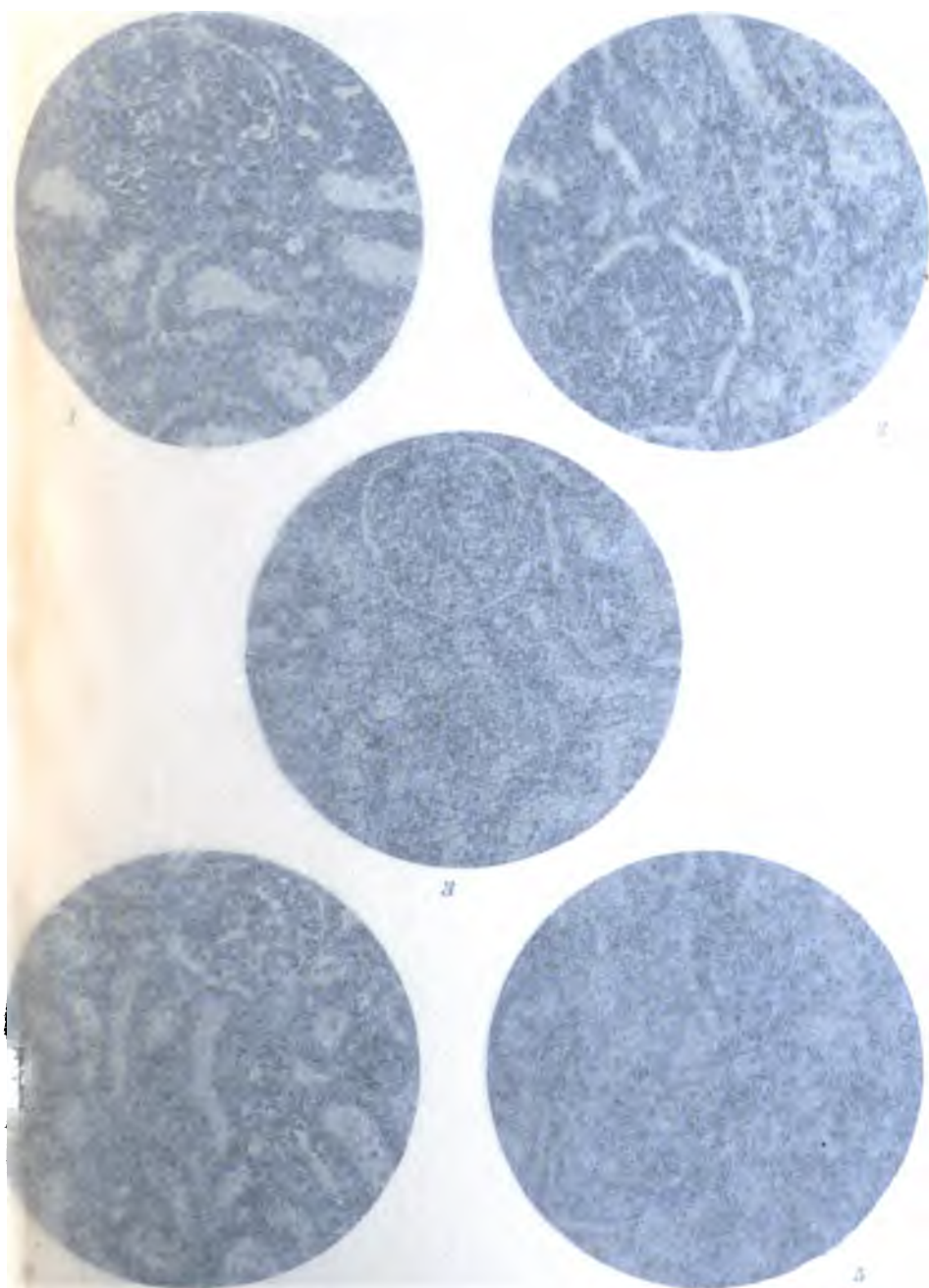
FIG. 16. — B. and L. obj. 3, oc. 1. Series 6, No. 13. The figure is from the kidney of the same animal with a cantharidin nephritis. The kidney shows a vascular nephritis with only slight changes in the epithelium. The output of urine was but slightly reduced.

FIG. 17. — B. and L. obj. 3, oc. 1. Series 8, No. 1. The figure shows the kidney in a late uranium nephritis with a histologically well preserved glomerulus. The epithelium shows the swelling and vacuolation which develops prior to necrosis. Urine greatly reduced.

FIG. 18. — B. and L. obj. 3, oc. 1. Series 8, No. 2. The figure shows the kidney of an animal anuric from uranium. The glomeruli are remarkably well preserved. The epithelium is necrotic and occludes the lumen of the tubules.

FIGS. 19 and 20. — B. and L. obj. 3, oc. 1. Series 8, Nos. 3 and 5. The figures represent the kidneys of animals nephritic and polyuric from uranium. The epithelium shows no encroachment upon the lumen of the tubules. The lumen of some of the tubules appears unusually large and in such tubules the epithelium appears shrunken.

FIG. 21. — B. and L. obj. 3, oc. 1. Series 8, No. 6. The figure represents the kidney anuric from uranium. The glomerulus is histologically well preserved. The epithelium shows advanced cloudy swelling and partial occlusion of the lumen of the tubules.



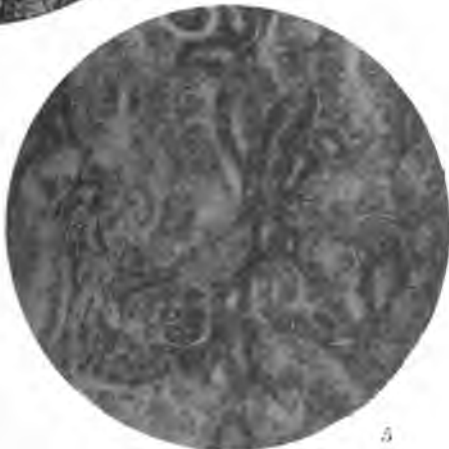
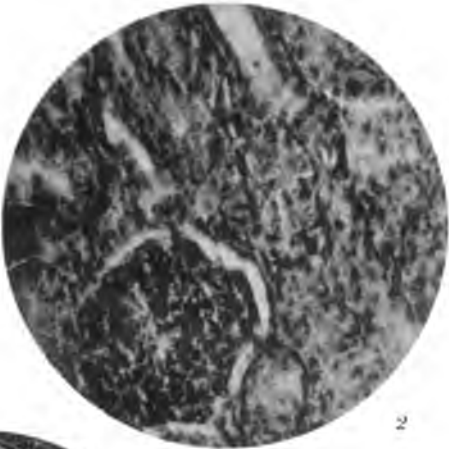
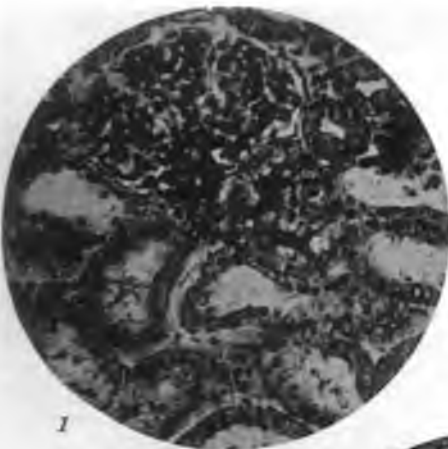
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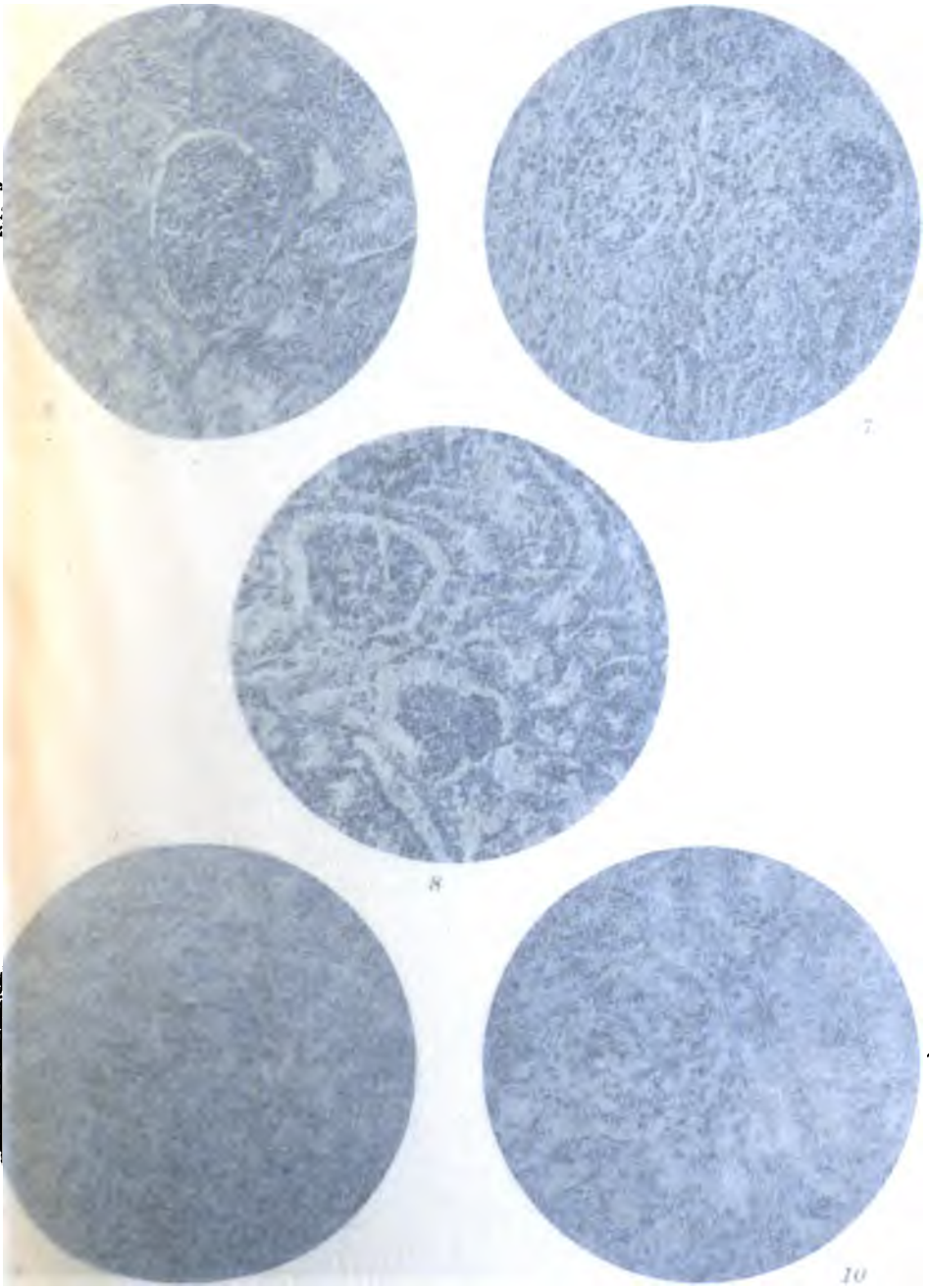
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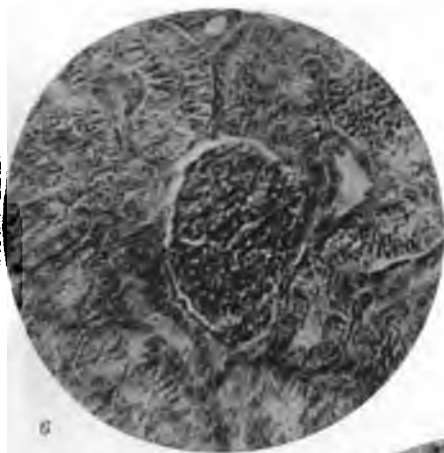
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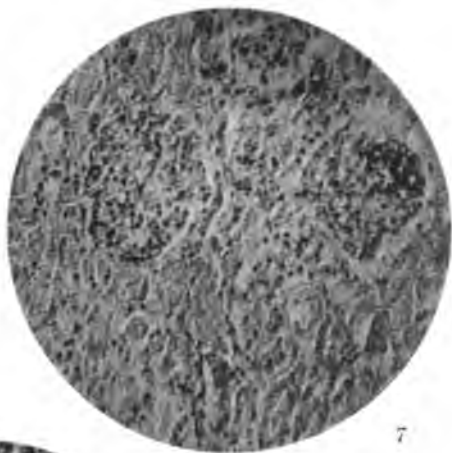
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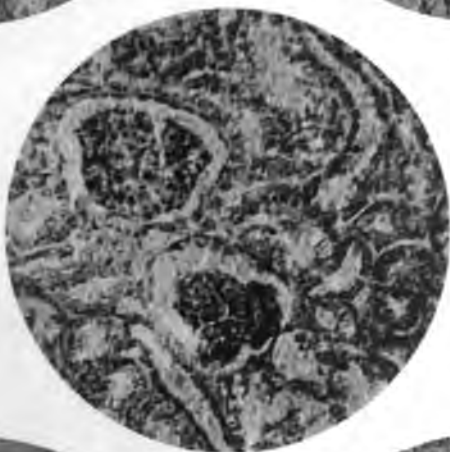




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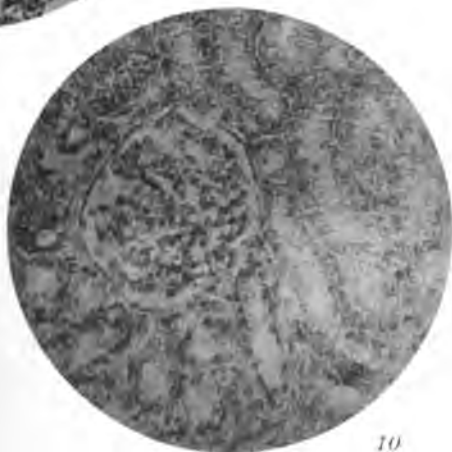
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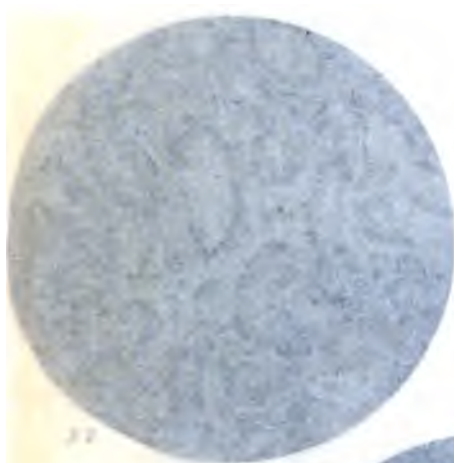
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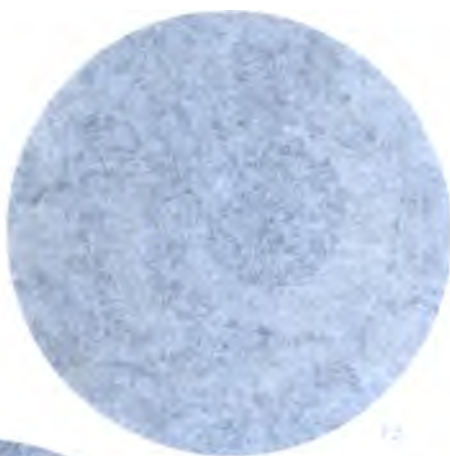
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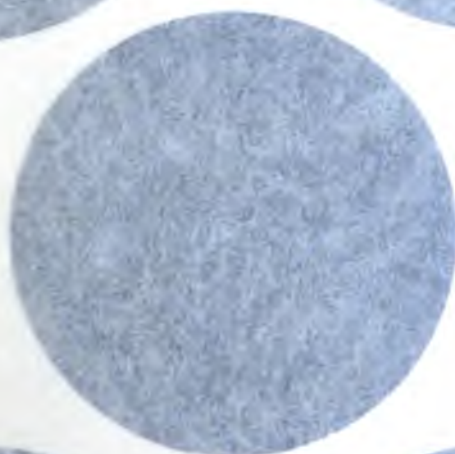
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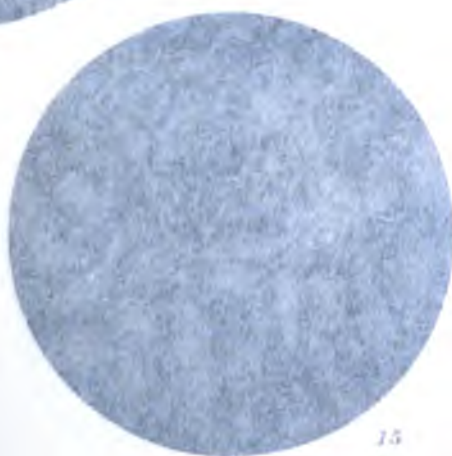
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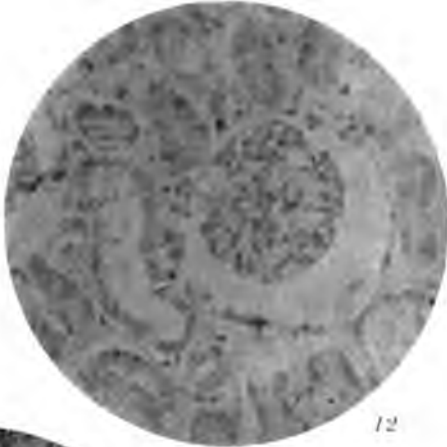


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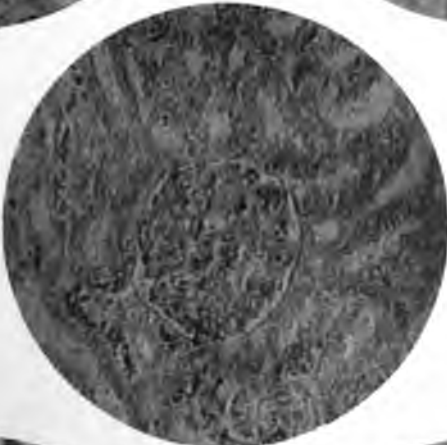
Experimental Results



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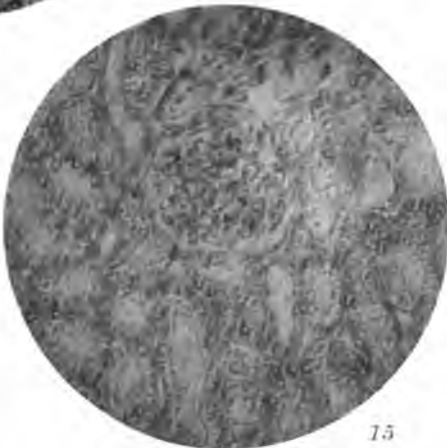
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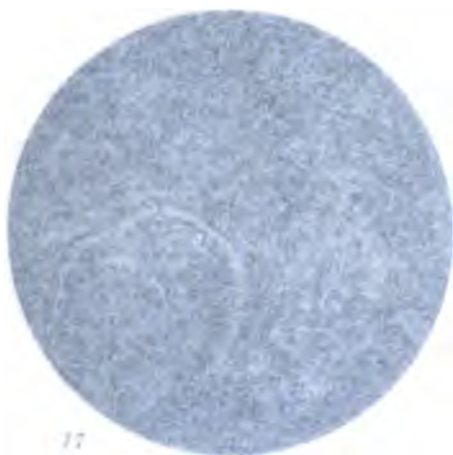
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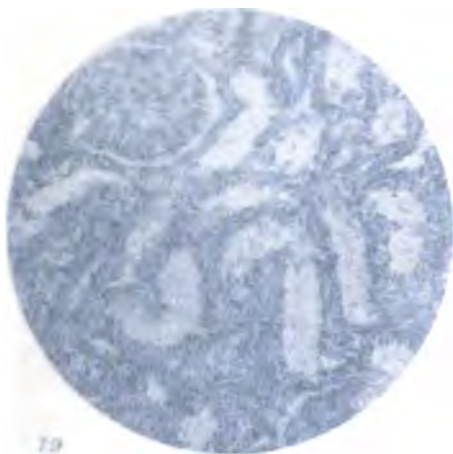
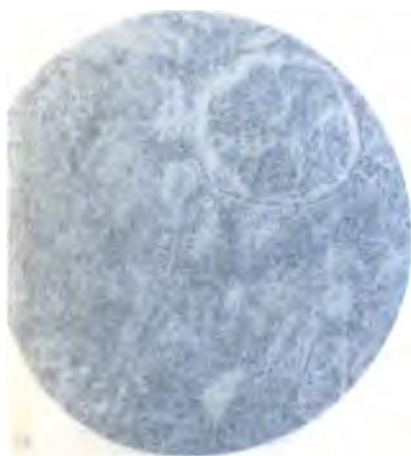
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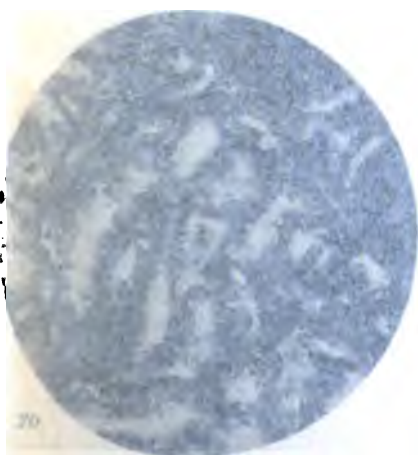
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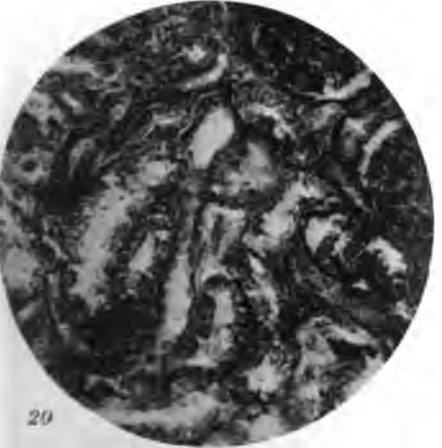
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TESTS OF THE EFFICIENCY OF PASTEURIZATION OF MILK UNDER PRACTICAL CONDITIONS.*

EDWIN HENRY SCHORER AND M. J. ROSENAU.

(From the Department of Preventive Medicine and Hygiene, Harvard.)

To the sanitarian, pasteurization has but one purpose — the destruction of pathogenic microorganisms. The thermal death points of the particular pathogenic microorganisms which sometimes invade milk have been determined with precision by many experiments in many laboratories. It has, however, been an open question whether the results obtained in the laboratory could be translated with safety to the pasteurization of large volumes of milk under commercial conditions. We are sure that 60° C. for twenty minutes is sufficient heat and time to destroy the viruses of non-spore-bearing microorganisms such as those causing tuberculosis, typhoid fever, diphtheria, and other infections which are sometimes found in milk. Biologically the problem has been solved, but the physical difficulties still remain; that is, it is difficult to heat a large volume of milk to a definite temperature and hold it at that temperature for a given period of time. It is a comparatively simple matter to accomplish this purpose with a small quantity of milk in a test-tube or flask under laboratory conditions. The difficulties are increased in practice by the fact that milk is a viscid fluid, and by the fact that the milk to be pasteurized varies in temperature as it flows to the heater. When milk is heated rapidly, as seems necessary under commercial conditions, the devices for regulating the temperature are neither sensitive enough nor quick enough to ensure the heating of all the milk to the desired temperature.

Few tests have been made upon the efficiency of pasteurization in actual practice for the reason that such tests are expensive and require an entire dairy plant at the disposal

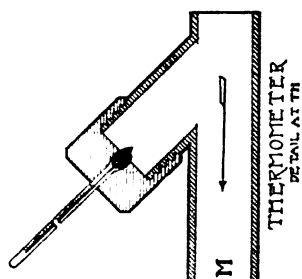
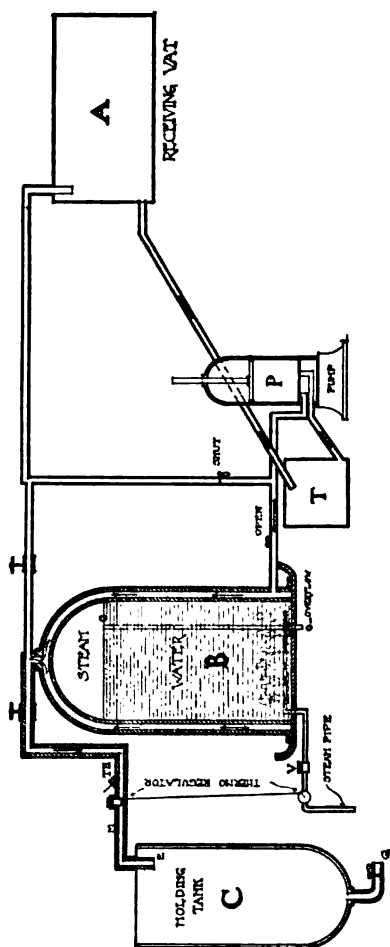
*This work was done under the auspices of the Milk and Baby Hygiene Association of Boston. Received for publication Feb. 15, 1912.

of the experimenters. We were fortunate in obtaining the conditions necessary to carry out these tests by having a dairy, equipped to handle a large volume of milk, placed at our disposal just before it was dismantled. We desire to express our appreciation to the proprietors of this plant, not alone for permitting it to be used, but also for furnishing about two thousand quarts of milk and other facilities necessary to carry out the tests.

Three methods of pasteurization are in use: (1) the flash method; (2) the holding method; (3) pasteurization in the bottle. The flash method, which is sometimes erroneously called "commercial" pasteurization, consists in momentarily heating the milk to a temperature of approximately 170° F. by allowing it to flow in a film over heated metal pipes or coils and then at once chilling it. The holding method consists in heating the milk to a temperature between 140° and 155° F. and then placing the heated milk in a receptacle where it is held at approximately this temperature for from twenty minutes to an hour. This was the method used in the experiments recorded in this paper. Milk pasteurized in the bottle according to the third method may be done at the home or at the dairy. Home pasteurization is particularly suitable for milk used in infant feeding. To pasteurize milk in the bottle upon a commercial scale, the bottle must be closed with a competent seal such as the crimped metal cap in common use. These bottles may then be immersed in a water bath, brought to the proper temperature and, after a suitable time, cooled. Another method of pasteurizing milk in bottles is in machines that have long been in use in the beer industry. In these machines the bottles are not immersed in water but are sprayed with a shower of hot water. Theoretically, pasteurization in the final container is preferable for the reason that the danger of subsequent contamination is eliminated, but the usual method of pasteurizing in bulk, in accordance with the holding method, has proven satisfactory in practice, especially when the milk is at once machine-filled into sterilized bottles.

Several indices may be used to determine the efficiency of pasteurization; (1) the time and temperature records; (2) the percentage reduction in the total number of bacteria, and (3) bacteriological tests to determine whether the pathogenic microorganisms have been killed or not. The first index is accurate and trustworthy provided the apparatus is so designed and managed that all the milk is thoroughly heated to the required temperature, and kept at that temperature for a sufficient length of time. From facts that are soon to be given it will be evident that the temperature readings, commercial thermometers, and thermal recording instruments are not always trustworthy. Thus we found in one of our tests that some of the milk flowing through an apparatus set at 145° F. only reached 123° F. The percentage reduction in the number of bacteria can only be taken as a general index that most of the milk has been sufficiently heated, for we have found that even with a reduction of ninety-nine per cent in the number of bacteria, the milk was so unevenly heated that some tubercle bacilli escaped. The third method of testing the efficiency of pasteurization, viz., by determining whether the pathogenic microorganisms have been destroyed, is too difficult and time-consuming to apply in routine practice, and is only applicable to special tests such as are submitted in this paper.

Description of pasteurizing plant. — The dairy plant placed at our disposal for the purpose of carrying out the tests recorded below was equipped to pasteurize 4,000 quarts of milk per hour in accordance with the holding method. The system consisted of four essential parts, — a receiving vat, a milk pump, a heater, and a holding tank. The details are shown in Fig. 1. The receiving vat (A) is a large metal receptacle with a capacity of about one hundred gallons. From the receiving vat the milk flows through a pipe to a smaller tank T, from which it is pumped through the heater B. The heater shown in Fig. 1 is the simple heater without the regenerating chamber, which is only provided for economic purposes and was not used in our experiments. The



dome-shaped heater is partly filled with water, which is kept at a constant level by means of the overflow pipe O. The water is heated by means of steam from a perforated pipe as shown in the diagram, and the space between the water and the dome of the heater is filled with live steam. The milk to be pasteurized is forced over this heated surface by the milk pump. After being heated the milk flows to the holding tank C, where it is held for the desired length of time.

In order to regulate the temperature of the heated milk an automatic device for increasing or decreasing the amount of steam is connected between the valve of the steam pipe and the thermoregulator upon the milk pipe M. In this way the amount of steam admitted to the heater B may be automatically regulated and controlled within certain limits. This mechanical regulator was not used in our experiments; in its place the temperature was controlled by hand. An experienced operator opened or closed the valve (V) as the temperature of the milk fell or rose.

As a further guide to the working of the heater a thermometer is inserted in the milk pipe M at a point Th. Upon closer examination it was found that the thermometer is placed in a dead end of pipe, and furthermore the bulb of the thermometer projects less than one-quarter of an inch into the milk in this dead end (see detail Fig. 1). Such a thermometer, therefore, does not record the temperature of all portions of the milk flowing through the pipe M.

Temperatures of continuously flowing milk may be taken in two ways: either a thermometer may be held in the flowing stream or else parts of the flow may be collected from time to time in an appropriate container and the temperature then taken of these portions. Obviously a thermometer in the stream gives, within certain limits, only the mean temperature and fails to record either the maximum or minimum temperature of certain parts of the milk that pass. The lag even in a delicate thermometer would here play a considerable part. In our experiments we always compared the temperature recorded upon the commercial type of thermometer in the milk pipe with frequent temperature readings

of portions of the flow collected in a quart measure, and tested with our standardized thermometer.

The actual temperature of the milk. — The efficiency of the apparatus placed at our disposal was tried in four tests or runs. In the first run it was planned to heat the milk to 145° F., in the second run to 140° F., in the third to 145° F., and in the fourth to 140° F. The temperature was regulated by hand; that is, an experienced operator opened or closed the valve V admitting steam to the heater. During each run temperature readings of the milk were frequently taken at various parts of the system. Observations were made every half minute, and more frequently in case of variation, of the temperature as recorded in the thermometer in the pipe M leading from the heater B to the tank C, Fig. 1. The temperature of the milk as it entered the tank C was taken by introducing a thermometer into the milk stream at e in Run I. These temperatures were taken as often as possible — the readings, however, were obtained with some difficulty owing to the vapor arising from the heated milk as it left the pipe e. Therefore in subsequent Runs II., III., and IV., the temperature of the milk flowing from e was taken by collecting portions of it in the quart measure.

The maximum and minimum temperatures of the milk in the holding tank C in Runs III. and IV. were taken with special thermometers which were placed at different levels. After the milk was held for the desired length of time the stop-cock g was opened. It was found that the first milk that flowed had a very much lower temperature than that desired; a considerable quantity of milk was permitted to run to waste in order to warm the pipe. After this, samples were collected and temperature readings made as fast as the quart measure could be filled and the readings taken.

The various temperatures recorded for the four runs are as follows:

Run I. — In this test the attempt was made to pasteurize one hundred gallons of milk at 145° F. for twenty minutes. The microörganisms to be tested (tubercle, typhoid, and

diphtheria) were suspended in salt solution and then added to one hundred gallons of raw milk in the receiving vat A, and thoroughly mixed by stirring. Meanwhile the heater B was warmed with water. As soon as the water reached the desired temperature (145° F.) the milk to be tested was run in and at the same time the water was allowed to run out. The temperature of the milk to be tested was under 50° F. As soon as this cold milk reached the heater there was an abrupt drop in the temperature of the heater and the first milk naturally did not reach the temperature desired. This first portion was therefore discarded. It took six minutes to run the entire one hundred gallons of milk tested through the heater. During this six minutes of heating sixteen temperature observations were made at the heater B and five as the milk reached the tank at e.

The holding tank had been previously warmed and the milk was held twenty minutes counting from the time the last portion reached the tank. It required six minutes to empty the tank and during this time thirteen temperature observations of the milk were taken, as shown in Table I. No temperature record was made of the first milk leaving the pipe g, for reasons already stated.

TABLE I. RUN I.

Temperature of milk taken at three different points in the system.

At Heater (B).	At Tank (c).	From Tank (g).
150° F.	131.0° F.	125.6° F.
149	134.6
147	136.4
145	141.8
138	123.2	140.9
147	123.2	143.6
148	144.5
149	145.4
150	145.4
152	144.5
158	146.4
159	146.0
157	163.4	149.0
155	
153	
150	156.2	

This run illustrated some of the difficulties encountered in rapidly heating a large volume of milk to a given temperature. Thus the milk as it left the heater varied from 138° to 159° F. Again, the temperatures of the milk as it reached the holding tank showed variations from 123.2° to 163.4° F. It will therefore be seen that the thermometer in the pipe leading from the heater does not catch either the maximum or the minimum temperature of all the parts of the flowing milk.

The milk in the tank itself also varied. The warmest milk naturally rose to the top and reached a temperature of 149° F., whereas the bottom layers were only 125.6° F.

Here it should be noticed that the bottom layers are not only the coolest portion of the milk, but may be the portions held for the shortest period of time.

Run II. — In this test an attempt was made to pasteurize one hundred gallons of milk at 140° F. for twenty minutes. The method of operating the pasteurizing machinery differed somewhat from that just described for Run I. and was as follows :

One hundred gallons of milk were placed in the receiving tank. Then the cultures to be tested (tubercle, typhoid, and diphtheria) were added to the milk. This infected milk was then passed through the heater and back to the receiving tank for the purpose of obtaining a uniform mixture and also for the purpose of warming it and the apparatus to the desired temperature. As soon as the entire bulk of milk was warmed to approximately 140° F., which required eight minutes, it was then diverted to the holding tank. The time required to fill the holding tank was three minutes, during which time six temperature observations were taken at the heater B, and three temperature readings of the milk as it flowed into the holding tank at c.

TABLE II. RUN II.
Temperature of milk taken at three different points in the system.

At Heater (B).	At Tank (c).	From Tank (g).
139° F.	128.3° F.
142	129.2
141	143.6° F.	130.1
135	131.0
139	134.6	131.6
139	140.9	131.9
		131.9
		132.3
		131.6
		132.3
		132.3
		132.3
		132.8
		132.8
		132.3
		132.8
		132.8
		133.2
		133.2
		133.2
		133.2
		133.6
		133.6
		133.6
		134.6
		134.9
		134.6
		134.6
		134.6

It will be seen from the above table that in this run the temperature of the milk at the heater varied from 135° to 142° F., a variation of seven degrees. More accurate temperature records taken from the milk as it flowed into the tank at e showed a variation of nine degrees, viz., from 134.6° to 143.6° F.

In the holding tank itself the bottom layers of milk had a temperature of only 128.3° F., while the upper layers reached 134.9° F., a difference of eleven and six-tenths degrees. The time of holding the milk was again determined in this run, as in the three other tests, from the time when the last milk reached the holding tank. It will be noticed that in this run, which permitted practically no factor of safety, the actual temperature of the milk fell seriously below that required to kill tubercle bacilli and other non-spore-bearing bacteria — 140° F. for 20 minutes.

Run III. — In this test the attempt was made to pasteurize one hundred gallons of milk at 145° F., holding some of it for twenty minutes and the remaining portion for forty-five minutes. The milk in this run was infected with massive cultures of bovine tubercle bacilli. The method of mixing and heating the milk was the same as that described for Run II. It required only two minutes to run the warmed milk through the heater to the holding tank. During these two minutes fifteen temperature observations were made at the heater B, and four as it entered the holding tank at e as shown in the following table:

TABLE III. RUN III.

Temperature of milk taken at three different points in the system.

At Heater (B).	At Tank (c).	From Tank (g).	
		20 Minutes.	45 Minutes.
146° F.	147.2° F.	132.8° F.	131.0° F.
146	136.4	134.6
147	136.4	134.9
146	137.3	136.4
145	149.0	137.6	136.4
144	138.2	136.4
144	136.7
145	137.3
146	143.6	137.3
147	137.3
147	137.8
148	137.8
148	138.2
147	138.4
145	143.6	138.4
			140.0

It will be seen from the above table that in this run the milk as it flowed through the heater varied only 4° F., viz., from 144° to 148° F. Further, it will be seen that the milk as it reached the holding tank, where more accurate observations were possible, the variation was only 5.4° F., viz., from 143.6° to 149° F.

The temperatures of the milk in the holding tank after being held twenty and forty-five minutes in the tank showed the same phenomena of layering; that is, the warmer milk rose, the difference between the top and bottom layers being

as much as nine degrees. A self-registering maximum thermometer placed in the milk in the holding tank at different levels after the first ten minutes showed a maximum reading of 141.4° F.

Run IV. — In this test the attempt was made to pasteurize one hundred gallons of milk at 140° F. for twenty minutes. In this experiment the milk was inoculated with massive cultures of bovine tubercle bacilli. The milk was mixed and warmed by the same method used for Runs II. and III. After the milk was brought to 140° F. it required three and three-eighths minutes to run the milk through the heater. During this time twenty-two changes of temperature were noted at the heater B, and eight as it entered the holding tank at e, as shown in the following table:

TABLE IV. RUN IV.
Temperature of milk taken at three different points in the system.

At Heater (B).	At Tank (e).	From Tank (g).
140° F.	147.2° F.	133.16° F.
145	134.9
144	135.1
144	141.8	136.0
143	136.0
142	140.0	135.8
141	136.4
140	136.4
139	143.4	136.9
140	136.8
141	136.8
142	141.8	137.0
143	137.0
144	136.8
143	137.5
142	136.2	137.84
141	137.84
140	135.2	137.84
138	137.8
137	136.4
139	136.4
140	136.4	

It will be seen from the above table that the temperature of the milk as it flowed through the heater varied from 137° to 145° F. — a difference of eight degrees. More reliable temperature readings taken of the free milk at the point e varied twelve degrees, viz., from 135.5° to 147.5° F.

After twenty minutes in the holding tank the temperature was only 133.16° to 137.84° F. The maximum temperature of the milk in the holding tank after seven minutes was 140° F.; after eighteen minutes 136.9° F.

TABLE V.
A condensed summary showing the variations in temperature for the four runs.

Run.	Temper- ature Desired.	At Heater (B).				At Tank (c).				After Holding (g).				Maximum Temperature.
		Highest.	Lowest.	Variation.	Average.	Highest.	Lowest.	Variation.	Average.	Highest.	Lowest.	Variation.	Average.	
I.	145° F.	159	138	21	150.8	163.4	123.2	40.2	139.64	149.0	125.6	13.4	141.94	10 minutes = 141.4° F. 7 minutes = 140° F. 18 minutes = 136.9° F.
II.	140° F.	142	135	7	139.16	143.6	134.6	9.0	139.64	139.96	128.3	11.6	132.69	
III.	145° F.	148	144	4	145.3	149.0	143.6	5.4	141.8	138.2 (a) 140.06 (b)	132.8	5.4 9.06	136.4 136.85	
IV.	140° F.	145	137	8	141.45	147.5	135.5	12.0	140.36	137.84	133.16	4.7	136.52	

(a) 20 minutes.

(b) 45 minutes.

It is now plain from the foregoing observations that considerable difficulty is met with in heating milk rapidly to a desired and uniform temperature using live steam as a heating medium. The temperature records of pasteurizing runs in working plants show, however, little variation. Further observations therefore were made at a larger dairy using a well recognized pasteurizer in first class condition to determine the accuracy with which automatic regulators control the temperature of the flowing milk. The following observations show temperature records taken at intervals of thirty seconds. These readings were made by catching a cupful of the milk, the temperature of which was taken with standardized thermometers at intervals of thirty seconds:

143.24° F.	144.32° F.	144.32° F.
143.96	143.24	143.58
143.58	142.76	141.44
142.76	143.24	142.52
143.24		

The greatest variations observed in this case were only from 141.44° to 144.32° F., a difference of 2.88° F. during a run of six minutes. It is to be observed that the above observations were made after the heater had been running for several hours and had settled down to a satisfactory automatic regulation. This, however, is not the case during the first seven or eight minutes after the apparatus is started, as will be seen from the following table, which shows the temperatures recorded at thirty second intervals during the first ten minutes with the same apparatus:

144.32° F.	132.60° F.	144.68° F.
141.80	131.70	143.60
147.70	142.76	144.32
141.80	143.96	143.60
141.80	143.96	142.12
138.20	145.76	143.96
136.40	143.96	144.50

It is therefore plain that the variations at first are considerable; in this case from 131.7° to 147.7° F., a difference

of 16° F. At least seven minutes is required to get the automatic apparatus under satisfactory working control. This difficulty in regulating is not to be lost sight of, for when the process of pasteurization has to be interrupted there is likely to be a multiplication of irregularities each time the heater is started anew.

In the holding tank the warmer milk rises and the cooler layers settle to the bottom. It is evident that the coolest portion of the milk may be held for the shortest time. To overcome this difficulty a series of holding tanks have been designed in which milk passes from one tank to the other. The tanks are cylindrical in shape. They are filled from the top and discharge from the bottom after they are full. The tanks and connecting piping are made of such size that with a maximum inflow it will require at least fifteen minutes to fill each tank. The first tank is provided with a feeding pan which has a feed outlet of the proper dimensions to permit the passage of such an amount of milk per hour as equals the rated capacity of the tank or series of tanks. In case a greater amount of milk is supplied than this rated capacity of the feed pan, the excess will pass through the overflow pipe that leads from this pan through the outside of the first tank. With this device it is therefore impossible to supply the milk at an increased rate and thereby shorten the time at which it is intended that the milk shall be held.

The first tank in such a series helps to equalize the temperature so that there is little variation in the temperature of milk flowing from it provided the tank is properly protected. The outflow from the second, third, and fourth tanks is quite uniform in temperature and in a well warmed room there is comparatively little loss of heat. This is well illustrated by the following observation:

The temperature of the milk discharged from the first tank when the heating varied from 141.44° to 144.32° F. was 142.66° F., from the second tank 140.54° F., from the third 139.8° F., and from the fourth 139.2° F.; the total loss of heat in the last three tanks being only 3.46° F. in forty-five minutes.

Pathogenic bacteria used in determining the efficiency of pasteurization. — The cultures used to test the efficiency of pasteurization in our work were the following: *B. diphtheriæ*, *B. typhosus*, *B. tuberculosis humanus*, and *B. tuberculosis bovis*. In each of the four runs made, one hundred gallons of milk were inoculated with one or more of these cultures.

Milk in Runs I. and II. were inoculated with *B. tuberculosis* (human), *B. diphtheriæ*, and *B. typhosus*.

Milk in Runs III. and IV. were inoculated with the bovine variety of the tubercle bacillus.

The cultures of the human variety of *B. tuberculosis* were obtained from the following sources: fifteen glycerine agar cultures from Dr. Theobald Smith, eleven potato cultures from Dr. W. H. Park, two glycerin agar cultures from our own laboratory. Of the cultures of the bovine variety of *B. tuberculosis*, seven cultures on glycerine agar were furnished by Dr. Smith, seven cultures on potato by Dr. Park, six cultures on glycerine agar by our own laboratory, and one by the Bureau of Animal Industry at Washington. Twenty-five different strains of *B. diphtheriæ* were obtained from Dr. B. L. Arms, Director of the Bacteriological Laboratory of the Boston Board of Health, and twenty-five agar slants of various strains of *B. typhosus* were obtained from a number of different sources. The cultures were uniformly suspended in salt solution. Approximately the same amount of culture material was added to the milk for Runs I. and II. The milk for Runs III. and IV. received equal amounts of the suspension of the bovine variety of *B. tuberculosis*. The suspension in each case was well mixed with the milk in the tank A.

Methods of determining the efficiency of the four runs. — The efficiency of any system or process of pasteurization should be judged by destruction or failure to destroy the pathogenic bacteria in milk. Because such examinations are time-consuming they cannot always be resorted to, and in their place the percentage reduction in the number of bacteria is ordinarily taken as the criterion on which efficiency is based.

In our investigation both the bacterial reduction and destruction of certain pathogenic organisms were studied and are here shown.

1. Bacterial reduction. — Agar plates in duplicate were made with definite quantities of milk to determine the number of bacteria per cubic centimeter in the milk just before and after pasteurizing. The results obtained from the four runs are as follows:

THE NUMBER OF BACTERIA PER CUBIC CENTIMETER IN THE MILK BEFORE AND AFTER PASTEURIZATION — PERCENTAGE REDUCTION.

Run.	Before Pasteurizing.		After Pasteurizing.		Efficiency.
	Individual Counts.	Average.	Individual Counts.	Average.	Percentage Reduction.
I.....	4,600,000 2,600,000	3,600,000	500 1,200	850	99.9%
II.....	6,700,000 9,600,000	8,650,000	19,000 17,000	1,800	99.9%
III.....	4,300,000 4,500,000	4,400,000	3,700 9,400	6,550	99.8%. Held 20 minutes.
			1,200 1,700	1,450	99.9%. Held 45 minutes.
IV.....	720,000 840,000	780,000	3,600 4,900	4,250	99.4%

The reduction in the total number of bacteria was marked in each trial, better in fact than is frequently accomplished by machines pasteurizing for the market, and according to this criterion any of the four runs should have been efficient. That this, however, was not the case is shown by the failure to destroy all the pathogenic bacteria in some of the test runs.

2. Destruction of pathogenic bacteria. — To determine whether the pathogenic microorganisms have been killed by the process of pasteurization is, after all, the crucial test —

for this represents the principal legitimate object of pasteurization. In the four tests which we made under practical conditions we made observations with the bacillus of diphtheria, the bacillus of typhoid fever and tubercle bacilli of both human and bovine types. In each run these organisms were again sought for in the pasteurized milk with the following results:

(a.) *B. diphtheriæ*. — The tests with *B. diphtheriæ* were carried out in Runs I. and II. In these runs pasteurization was attempted at 145° F. and 140° F. respectively. It should here be recalled that the temperatures attempted were not, in fact, attained.

One hundred gallons of raw milk were inoculated with fresh growths from twenty-five different strains of *B. diphtheriæ* on Loeffler's blood serum. The surface cultures were suspended in salt solution and then thoroughly mixed in the milk. From the milk thus inoculated two hundred cubic centimeters were centrifugalized and the cream and sediment and also the mixed milk were examined both by direct microscopic and also by cultural methods. Loeffler's blood serum and also human blood agar were the culture media used. Similar smears and cultures were made from the milk after pasteurization. In each case a large number of cultures were prepared. The results obtained are shown in the following table:

RUN I. — The pasteurization temperatures noted were the following:

At heater: Highest, 159° F.; lowest, 138° F.; average, 150.8° F.

At tank: Highest, 163.4° F.; lowest, 123.2° F.; average, 139.64° F.

After holding 20 minutes: Highest, 149.0° F.; lowest, 155.6° F.; average, 141.94° F.

	Before Pasteurization.			After Pasteurization.		
	Stain.	Loeffler.	Human Blood.	Stain.	Loeffler.	Human Blood.
Cream	Occasional.	+	+	—	—	—
Sediment . . .	Few.	+		—	—	—
Milk	One in 3 fields.	+	—	—	—	—

From the above table it will be seen that diphtheria bacilli were killed in this test run.

RUN II. — The pasteurization temperatures were the following :

At heater: Highest, 142° F.; lowest, 135° F.; average, 139.16° F.

At tank: Highest, 143.6° F.; lowest, 134.6° F.; average, 139.64° F.

After holding 20 minutes: Highest, 139.96° F.; lowest, 128.3° F.; average, 132.62° F.

	Before Pasteurization.			After Pasteurization.		
	Stain.	Loeffler.	Human Blood.	Stain.	Loeffler.	Human Blood.
Cream	—	+	+	—	+	+
Sediment ...	Few.	Few.	—	—	+	+
Milk.....	One in 3 fields.	+	+	—	+	+

From the above table, which summarizes the results, it will be seen that some of the diphtheria bacilli survived. This run was planned at 140° F. for twenty minutes. As a matter of fact the temperature was considerably less. The results obtained clearly show the defects in apparatus of this kind under the conditions of the experiment. These conditions represent the defects which are found in machines of this type during the first five or seven minutes of their operation. It further emphasizes the necessity for a liberal factor of safety, in both temperature and time of holding.

(b.) *B. typhosus*. — Tests upon *B. typhosus* were determined in Runs I. and II. The same one hundred gallons of milk used for the preceding diphtheria tests were also inoculated with *B. typhosus*. The fresh young growth upon twenty-five tubes of slanted agar were suspended in salt solution and thoroughly mixed in the milk. Typhoid colonies were searched for both before and after pasteurization by cultural methods from two hundred cubic centimeters of the milk which was centrifugalized for this purpose. Both the cream

and the sediment, as well as the whole milk, were inoculated upon the surface of Petri dishes containing Endo's medium. These plates were incubated at 37° C. and after twenty-four and forty-eight hours all colonies resembling those of *B. typhosus* were transferred to Hiss' semi-solid medium; organisms showing motility and not producing gas were tested out upon differential media, and finally the cultures were tested for agglutination with a specific serum. The results obtained are shown in the following table:

RUN I. — The temperatures noted were the following;

At heater: Highest, 159° F.; lowest, 138° F.; average, 150.8° F.

At tank: Highest, 163.4° F.; lowest, 123.2° F.; average, 139.64° F.

After holding 20 minutes: Highest, 149° F.; lowest, 125.6° F.; average, 141.94° F.

	Before Pasteurization.	After Pasteurization.
Cream	+	—
Sediment	+	—
Milk.....	+	—

It will be seen from the above table that the heating was sufficient to kill *B. typhosus* so far as could be determined.

RUN II. — The temperatures noted were the following:

At heater: Highest, 141° F.; lowest, 135° F.; average, 139.16° F.

At tank: Highest, 143.6° F.; lowest, 134.6° F.; average, 139.64° F.

After holding 20 minutes: Highest, 139.96° F.; lowest, 128.3° F.; average, 132.62° F.

	Before Pasteurization.	After Pasteurization.
Cream	—	+
Sediment.....	+	—
Milk.....	+	+

From the above table it will be seen that the typhoid bacillus survived the process of pasteurization in Run II. Diphtheria bacilli also survived in this run. Attention is again called to the fact that although the experiment was planned at 140° F. for twenty minutes, the actual temperature fell seriously below that desired.

(c.) *B. tuberculosis*. — In addition to the direct microscopic examination of stained smears a number of guinea-pigs were injected subcutaneously with the cream and sediment obtained from the raw milk. The cream and sediment were obtained by centrifugalizing two hundred cubic centimeters of a representative sample. As a control twenty-one guinea-pigs were injected subcutaneously with five cubic centimeters each of the sediment and twenty-two guinea-pigs were injected subcutaneously with five cubic centimeters each of the cream, obtained from the raw milk.

Similar tests were made with the milk after pasteurization. Two hundred cubic centimeters were centrifugalized and twenty-nine guinea-pigs were injected subcutaneously with five cubic centimeters each of cream and twenty-eight guinea-pigs were injected subcutaneously with five cubic centimeters each of sediment.

As a further control of the virulence of the cultures used, ten guinea-pigs were injected subcutaneously with suspensions in salt solution. These suspensions were made to approximate as closely as possible the number of tubercle bacilli in the milk tested; that is, one suspension contained approximately two tubercle bacilli of the human type to each oil immersion field, and the other suspension five tubercle bacilli of the bovine variety to each field.

Some of the guinea-pigs died of acute infections and were therefore lost so far as the object of our experiments were concerned. Some of the animals died of generalized tuberculosis. Those that did not die were tested with tuberculin. All the animals were finally autopsied and examined for evidences of tuberculosis. Smears were made from the various organs and tissues, and histological studies were

undertaken in all cases in which the diagnosis was doubtful. The smears were stained by the usual method, acid and alcohol being used to decolorize. The organs and tissues for histological examination were fixed in Zenker's fluid, imbedded in paraffin, and sections stained with hematoxylin and eosin.

Realizing that the heat might attenuate the tubercle bacilli or so influence them as to give atypical lesions, and further realizing that the presence of dead bacilli in the tissues might complicate the situation, secondary guinea-pigs were inoculated with material from doubtful cases. In some instances the tissues and organs from these secondary pigs were further transferred to other pigs.

In a number of guinea-pigs a negative diagnosis of tuberculosis was made at the autopsy despite the fact that acid and alcohol-fast bacilli were seen in the smears from the inguinal and axillary glands. In these cases the glands, as well as other tissues and organs, were injected into secondary guinea-pigs. In addition a particularly searching histological study was made of such glands and tissues. The fact that organisms resembling tubercle bacilli were found in the superficial lymph nodes, but further tests failed to disclose evidence of tuberculosis, was interpreted by us to mean that dead tubercle bacilli, injected subcutaneously with the heated milk, were simply caught in these structures. All the tests were not made in every negative case, especially if there were positive pigs in the same series. However, all the tests were made in every case in which the series was negative in any particular run.

On the other hand, some guinea-pigs in which no particular evidence of tuberculosis could be seen with the naked eye at autopsy, were further tested with the result that live tubercle bacilli were demonstrated.

In brief, the results obtained with tubercle bacilli may be summarized as follows:

1. Controls in salt solution. — All of the guinea-pigs injected with the suspension of the human and bovine

variety of tubercle bacilli died either of generalized tuberculosis or died after an injection of tuberculin. All the controls showed definite evidences of tuberculosis. The culture material used was therefore virulent for guinea-pigs.

2. Controls in cream. — Twenty of the twenty-nine guinea-pigs injected with the cream died of mixed infections and therefore were lost for the purpose of these experiments. Nine of the guinea-pigs overcame these early infections and subsequently developed tuberculosis. Some of them died of generalized tuberculosis; others died after an injection of tuberculin. Evidences of tuberculosis were demonstrated in each case.

3. Controls in sediment. — Twenty-eight guinea-pigs were injected with the sediment obtained by centrifugalization. Twelve died of mixed infections; the remaining sixteen developed definite evidences of tuberculosis.

It is of interest here to note that a much higher percentage of the animals injected with the cream died of early mixed infections than those injected with the sediment. A larger amount of cream than sediment was injected into the guinea-pigs — which may account for this difference.

4. Results of the heating upon the tubercle bacilli in the milk.

RUN I. — Human variety of *B. tuberculosis*. The temperatures noted were the following:

At heater: Highest, 159° F.; lowest, 138° F.; average, 158.8° F.

At tank: Highest, 163.4° F.; lowest, 123.2° F.; average, 139.64° F.

After holding 20 minutes: Highest, 149° F.; lowest, 125.6° F.; average, 141.94° F.

	Before Pasteurization.					After Pasteurization.							
	No. Injected.	Cause of Death.			Result.	No. Injected.	Cause of Death.			Result.			
		Mixed Infec- tion.	Tuber- culosis.	Chloro- formed.			Mixed Infec- tion.	Tuber- culosis.	Chloro- formed.				
Cream	5 (Nos. 1-5)	5			+	—	6 (Nos. 10-15)	1	1	2	3 1 (?)	1	
Sediment	4 (Nos. 6-9)	3	1			1	6 (Nos. 16-21)	0	0	0	6	2	4

Sub-inoculations were made from two guinea-pigs (Nos. 10 and 13) injected with cream. The animals injected from guinea-pig No. 10 gave negative results and those injected from No. 13 died with mixed infection.

It is evident that pasteurization in this test run was not efficient in killing tubercle bacilli of the human variety.

RUN II. — Human variety of *B. tuberculosis*. The temperatures noted were the following:

At heater: Highest, 142° F.; lowest, 135° F.; average, 139.16° F.

At tank: Highest, 143.6° F.; lowest, 134.6° F.; average, 139.64° F.

After holding 20 minutes: Highest, 139.96° F.; lowest, 128.3° F.; average, 132.62° F.

	Before Pasteurization.					After Pasteurization.				
	No. Injected.	Death.			Tuberculosis.	No. Injected.	Death.			Tuberculosis.
		Mixed Infection.	Tuber. culin.	Chloro. formed.			Mixed Infection.	Tuber. culin.	Chloro. formed.	
Cream.....	5 (Nos. 22-26)	5				4 (Nos. 33-35)	1	1	1	3
Sediment.....	5 (Nos. 27-32)	3	1	1	2	4 (Nos. 36-39a)		1	3	2
										1 1 (?)

Sub-inoculations were made from Nos. 32, 38, and 39a. Of these the animals injected with material from Nos. 32 and 38 were definitely tuberculous.

Pasteurization in this test run was not efficient in destroying the tubercle bacillus of the human variety.

RUN IV. — Bovine variety of *B. tuberculosis*. The temperatures noted were the following:

At heater: Highest, 145° F.; lowest, 137° F.; average, 141.45° F.

At tank: Highest, 147° F.; lowest, 135.5° F.; average, 140.36° F.

After holding 20 minutes: Highest, 137.84° F.; lowest, 133.16° F.; average, 136.52° F.

	Before Pasteurization.				After Pasteurization.			
	No. Injected.	Death.		Tuberculosis.	No. Injected.	Death.		Tuberculosis.
		Mixed Infection.	Tuber. culin.	Chloroformed.		Mixed Infection.	Tuber. culin.	
Cream.....	6 (Nos. 74-79)	6			6 (Nos. 86-91)	0	1	3 1*
Sediment	6 (Nos. 80-85)	3	2	1	3	0	2	5 2(?)

* One guinea-pig lost from cage.

From this it will be seen that pasteurization in this test run was not efficient in killing the bovine variety of the tubercle bacillus.

.; average, 136.4° F.
 average, 136.85° F.

After Pasteurizing 45 Minutes.						
ected.	Death.				Tuberculosis.	
	Mixed Infection.	Tuber. culosis.	Tuber. culin.	Chloro-formed.	+	-
Cream b-69)	3	1		4		4 1(?)
Sedim -73)	0			4		4

ing tuberculous lesions. From one of these further sub-injections
 were a
 ly negative.

ing for twenty minutes, however, was not efficient
 to de
 milk
 sea-pigs injected with cream or sediment of such

SUMMARY AND CONCLUSIONS.

To the sanitarian, pasteurization has but one purpose — the destruction of pathogenic microorganisms. Biologically, the problem has been solved, but some of the physical difficulties remain. It is not a simple matter to heat a large volume of milk to a definite temperature and hold it at that temperature for a given period of time.

When milk flows rapidly over a steam-heated surface the milk is not heated uniformly. Unequally heated milk placed in a holding tank does not necessarily mix but tends to arrange itself in layers. Therefore, milk heated rapidly by passing over a steam-heated surface should be equalized by mixing in a tank before it goes to the holding tank. In most commercial pasteurizers the heating surface is too limited, the flow too rapid, and the holding tanks either too small or too few in number to insure uniform and trustworthy results.

We undertook tests to determine the effectiveness of pasteurization under the conditions which commonly obtain in commercial practice. The tests were made at a large dairy plant which was placed at our disposal just before it was dismantled. The essential parts of the pasteurizing apparatus consisted of a heater and a holding tank.

Four separate tests were made, each with one hundred gallons of milk.

In two of the tests it was planned to heat the milk to 140° F. and in the other two tests to 145° F.

The temperature attempted was not attained. It is difficult to maintain and control the temperature of the milk when live steam is used as the heating medium. In some of the runs the actual temperature at times fell seriously below that desired and the pathogenic microorganisms were not all killed. In the first test the milk flowing from the heater varied as much as 21° F.; more reliable records obtained at the holding tank showed a variation of as much as 40.2° F.

In the first test in which it was attempted to pasteurize the milk at 145° F. some of the milk registered only 123.2° F.

We found that it requires about seven minutes for a pasteurizing heater, of the type used, to settle down to a

satisfactory working basis. That is, an automatically controlled pasteurizer does not heat the milk uniformly until it has been running at least seven minutes. The difficulty in regulating the temperature at the beginning of a run has considerable practical importance. In practice, pasteurization is frequently interrupted, especially when milk trains are late, and in such cases there is a multiplication of irregularities each time the process is started anew. Our four tests represented the conditions at their worst; that is, they are a precise parallel of the irregularities met with in starting a pasteurizing plant. We therefore recommend that milk which is pasteurized during the first ten minutes should again be run through the heater.

Obviously a thermometer in a stream of flowing milk gives, within certain limits, only the mean temperature, and fails to record either the maximum or minimum temperatures of parts of the milk that pass. The lag, even in a sensitive thermometer, is considerable. We have found that the thermometers sometimes are placed in dead ends of pipe, the bulb does not project sufficiently into the milk stream, and that other sources of error exist.

In a deep holding tank the warmer milk rises. The coolest layer at the bottom may be the portion held for the shortest period of time. In any holding device the time should be counted from the moment the last milk reaches the holder. The phenomenon of layering was so pronounced in one of our tests that a difference of 13.4°F. was observed between the top and bottom layers. We realize that this is exceptional and that such a great difference does not take place after the heater had settled down to a continuous working basis.

Automatic devices for thermo-regulation should be carefully tested from time to time and controlled with actual temperature observations of the milk with standard thermometers.

The principal legitimate object of pasteurization is the destruction of pathogenic microorganisms. We therefore

determined the efficiency of the apparatus with microorganisms that represent the most frequent and the most serious causes of trouble in milk, viz., tubercle, typhoid, and diphtheria.

In two tests with *B. diphtheriæ* one succeeded, but the other failed to kill all these bacilli which had been added to the milk.

In two tests with *B. typhosus* one succeeded, but the other failed.

In two tests with *B. tuberculosis* (human) both failed, and in two tests with *B. tuberculosis* (bovine) one succeeded and one failed.

Quantitative determinations of the number of typhoid, diphtheria, and tubercle bacilli that may have been killed were not made, but our results plainly indicate that there was a quantitative reduction in the case of the experiments with tubercle bacilli. That is, in the tests in which the pasteurization failed to kill tubercle bacilli it was evident that the failure was not total and that only some of the bacteria survived. Fewer of the guinea-pigs that were injected with the heated milk developed tuberculosis than the control pigs injected with equal quantities of the raw milk. Further, the tuberculosis in the guinea-pigs injected with heated milk was milder, more localized and more atypical than in the control animals. From this we conclude that many of the bacilli were killed or attenuated at least.

Nothing in our experiments throws doubt upon the thermal death points of the microorganisms tested. We are sure that if the milk reaches 140° F. and is held there for twenty minutes it will kill tubercle, typhoid, and diphtheria bacilli. Our experiments show that milk pasteurized at this temperature for the specified time may not always, in practice, reach these minimum requirements. It is therefore evident that a liberal factor of safety is necessary in the operation of this type of pasteurizer under commercial conditions. We further believe that the process should not be unduly hurried, for this increases the physical difficulties of uniformly heating all portions of the milk.

Perhaps the best temperature to meet practical conditions is 145° F., and the milk should be held at this temperature from thirty to forty-five minutes. This would give sufficient leeway. If the pasteurizer is set at 145° F. care will probably be taken that it does not go above 148° F., on account of destroying the cream line, and it is not very likely that the mixed milk in the holding tank would drop below 140° F., which is the minimum.

Our observations indicate that a single holding tank is not as trustworthy as two, three, or four in series. The first holding tank acts as an equalizer and the remaining tanks hold the milk at a uniform temperature for a definite period of time.

Our studies again emphasize the necessity for official control over all pasteurizing plants. The process is such an important public health measure that it should not be left to the caprice of the individual. When pasteurizing apparatus is run by rule-of-thumb methods there is danger of underheating and false security. It is surely just as important to standardize and guard the devices controlling the temperature of pasteurizing apparatus as it is to watch weights and measures.

SOME OBSERVATIONS ON THE CULTIVATION OF TISSUES
IN VITRO.*

G. C. WEIL.

(*The R. B. Mellon Fellow in Pathology.*)

(*From the Pathological Laboratories, University of Pittsburgh.*)

Through the work of Harrison of Yale, who demonstrated that tissues could be cultivated in media outside of the living body, a new impetus has been given to various cytological studies. In his early work Harrison demonstrated that portions of tissue isolated from embryonic nervous structures could be grown in the fluid obtained from the lymph sac of the frog. These implanted tissues showed, after several days, a development of prolongations from the isolated cells into the surrounding media. The cultures were prepared aseptically upon hanging-drop slides. Harrison found considerable difficulty in preventing bacterial infection of his cultures. He further found that although he attempted to deal with the nervous tissues from the medullary tube, in some cases portions of other tissues were also included in the preparation. This transplantation of several types of tissue led to some difficulty in indicating with any degree of accuracy the type of cell which showed proliferation in the prepared cultures.

The study of this material was confined, in the greater part, to observations made upon the nervous system. He found that the ordinary histological methods of dealing with adult tissues were inapplicable, owing to the extreme delicacy of the structures. For these reasons it was not possible to carry out the ordinary staining methods which are applied in the examination of adult tissues for the differentiation of particular types of cells or fibrils. Harrison confined his staining methods mainly to alcoholic hematoxylin. He found much difficulty in isolating and preserving nerve fibers which had developed in his artificial media.

The technic of Harrison was later modified by Burrows and

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the experiments were extended to other animals. Harrison found that the lymph from the frogs was not a very suitable medium to work with, as the coagulation of this fluid was not uniform. At times the lymph formed no clot or was much too soft to be easily handled. Burrows was able to substitute the blood plasma of frogs for the lymph. Later he was able to carry out similar experiments in using the plasma of chickens and inoculating this with the tissues from embryonic chicks. In using this method for the study of cell growth it was easier to carry out the aseptic technic than was possible with the frog preparations.

More recently this work has been still further extended by the experiments of Carrel and Burrows. These investigations have shown that by using a homologous plasma various tissues of embryonic and adult animals can be cultivated with fair ease. These authors claim to have grown connective tissue, cartilage, peritoneum, bone marrow, bone, skin, cornea, mucus membrane of the tongue, thyroid gland, spleen, suprarenal gland, kidney, pancreas, testicle and ovary from the dog and cat. At a later date they have reported the successful cultivation of human and fowl sarcoma upon the homologous plasma.

In the variety of organs from which authors obtained growth of cells, they claim they have observed the development of structures peculiar to these organs, such as tubules of the kidney. These authors were also able to obtain several generations of their cultures by transferring some of the material under cultivation to fresh media. They have also indicated that the cultivated tissue from fowl sarcoma will reproduce the tumor when inoculated into a healthy chicken.

Lambert and Hanes have used the method of cultivating tissue in vitro for studying cytolysins in connection with mouse and rat sarcoma. They obtained the interesting result that the mouse sarcoma grows vigorously in the plasma of normal rats, and that rat sarcoma is readily cultivated in the plasma of normal guinea-pigs. These studies indicate the important facts that tissues may live and grow

in heterologous plasma. This, as we shall point out later, has been our own finding, in that we were able to cultivate guinea-pig and chick tissues in human plasma.

The development of these methods for cultivating tissues outside of the body suggests the solution for many problems in immunity and cytology. However, much has yet to be done in the development of a technic by which certain definite and recognized tissues may be segregated and individually planted upon media without the intermingling of cells of a different character. Besides this a new histological technic is required by which we may use, with some security, stains which will differentiate recognized tissues in the embryonic state which do not take the microchemical reaction as do the adult tissues. It is found, for instance, that the early embryonic connective tissue cells do not show fibers by the selective staining methods as do the fibroglia of the adult tissues.

In our own work, to be described, we have found the greatest difficulty, not in the cultivation of tissues from different organs, but in the determination of the nature of the cells which are reproduced. As is recognized by histologists, the differentiation by staining methods of various types of cells when these have been isolated from their surrounding structures, from other cells of an entirely different origin, is extremely difficult, and the difficulty increases greatly when we are dealing with the differentiation of embryonic or actively growing cells. Up to the present a definite differentiation of cultivated tissues has not been made, nor have the various features of evolution of the embryonic cells of any type been followed through to the mature stage of that cell.

Technic. — In our efforts to obtain actively growing cultures we have found certain things of great importance, and in order to obtain the best results certain rules of technic must be closely followed. Strict asepsis during the manipulation as mentioned by previous writers has been found essential. While not producing immediate death, yet the presence and growth of bacteria in a great measure retards and limits the life of the growing culture. Nevertheless the

extent of harmful action depends largely upon the type of tissue and nature of the media used. In guinea-pig cultures we have found that various infections rapidly terminate the growth of the culture, while infection of chick tissue in chicken plasma simply retards and limits the growth. We have not studied the nature of these contaminating organisms.

In a general way our technic for preparing and inoculating media with living tissues differs little from that described by others, but a few points of importance in this respect may be worth mentioning. Tissues which are planted directly from a living animal or immediately after the death of the animal give the best results. It is essential to transfer these prepared cultures to the incubator immediately after they have been made and under these conditions evidence of growth is obtained in one and a half hours. It has also been found that tissues which have been teased from larger masses by means of sharp pointed instruments, just at the moment of inoculation, show a greater activity and respond more rapidly to growth.

For various purposes of experiment and observation we have selected different types of cultural methods. For observing, from time to time, the character and nature of the growing tissues on the warm stage, hanging-drop preparations have proved most convenient, while these preparations were also best adapted for purposes of staining and study of microchemical reactions.

In order to observe growing cultures macroscopically and for the purpose of transplantation, large plate cultures have been found best suited. The ordinary petri dishes, heavily coated about their margins with sterile vaseline or paraffin, are most convenient for this work. In this way considerable nourishment may be provided to the tissue, and under proper conditions a very luxuriant growth is obtained. The new growth may easily be distinguished by the naked eye in the halo effect or outer translucent margin as compared with the central opaque area of the original implanted mass.

In our selection of culture media we have found that

different types of tissue grow best in different media. As mentioned by Burrows and later by Lewis, connective tissues grow best in the homologous plasma, and while we have observed epithelial-like cells in plasma media they have been found to grow more abundantly in an agar serum medium.

In using plasma media, only a sufficient amount for immediate use is prepared, and while it may be preserved for some time in a liquid state at a low degree of temperature it was found that the longer it was kept the more did it inhibit active growth. In order to simplify our technic and to secure better results, fresh serum was always used. The plasma was obtained from the blood of various animals by withdrawing a sufficient quantity of fluid from the veins by means of a sterile syringe. This process was carried out under all aseptic precautions and without the use of an anesthetic, which we believe would interfere with the quality of the serum.

The blood thus collected is received in paraffined tubes and chilled. The blood is then rapidly centrifugalized without taking any particular precautions for keeping the blood cool other than that the centrifuge be in a cool place. With an adequate centrifuge the clear plasma may be collected within two minutes. This clear fluid is then transferred by means of paraffin tubes into containers chilled in ice. Such plasma will remain fluid for hours and is easily handled for the purpose of making cultures.

It is always well to have on hand the materials and instruments necessary for carrying on the culture before the plasma is prepared. The plasma is then handled in paraffin coated pipettes and spread upon cover-slips or plates upon which the culture is to be made. The plasma which is placed upon the cover-slips will usually remain fluid for a few minutes, and should be immediately inoculated before the fibrin has formed. Thin cultures are best in that they afford better opportunities for staining and growing the cultures and are more easily examined under the microscope.

It is well to note in the preparation of plasma cultures

that the coagulation time in different species varies somewhat, as for instance guinea-pig blood, even after thoroughly chilling, if left too long in the centrifuge, will develop a firm fibrin clot, while on the other hand chicken plasma does not show this tendency towards rapid clotting.

Serum collected in the ordinary way has also been used, but with only fair success. It was found that better results were obtained when the fibrin was present in the culture media. Probably the fibrin assists the growth in presenting a framework upon which the cells may develop, as well as affording useful nutriment when it is dissolved by the proteolytic ferment. In fluid serum fair growths of connective tissue were obtained, and these were always observed to be attached to the surface of the cover-slip.

Serum agar cultures were also prepared, one per cent plain agar was prepared with tap water without the addition of bouillon. This was put up in small tubes and when required was melted and again cooled to 40° C., when blood serum was added in varying dilutions. The best results were obtained when the serum and agar were mixed in the proportions of two to one or three to one. In this medium was observed a luxuriant growth of not only connective tissue fibrils but also epithelial-like cells. Bouillon was later added in various strengths to this serum agar mixture but with no distinct advantage. Carrel and Burrows were unable to obtain cultures on any serum media.

Staining. — The main difficulty which confronts the observer, after he has been successful in obtaining growths in his artificial media, is to apply a proper technic so that his cultures may be studied at leisure and that they may be kept for future reference and comparison. We have found that for the closer observation of cell structure it was best to stop the growth at the end of five days, before the degenerative changes altered the character of the cells. It was not uncommonly found that after six days there were various types of granules and globules within the cells, which, as we

will indicate later, are an evidence of a lowering of the cell vitality.

Moreover, it is very necessary to obtain suitable methods by which a staining technic could be applied to differentiate cells of different orders. As has been reported by other observers it is impossible to observe mitosis of cells in the living cultures. We have on repeated occasions closely watched the indefinite nucleus, but were never able to observe any arrangement of the chromatin filaments. That mitosis occurs is only to be indicated in stained preparations.

It is also to be noted that the cells developing in cultures react somewhat differently from the adult tissues in the animal body. It was commonly observed that in cultures where the connective tissue had been most active in proliferating and in which the general appearance of the cells and the arrangement indicated a connective tissue character of the structure, we were unable to get the differential staining reactions for connective tissue fibrils. At times these fibrils were wanting and the cells possessed only coarse protoplasmic processes.

In general we have found that formalin fixation was the best. The small hanging-drop preparation was immersed in five per cent formalin and allowed to fix for about an hour. It was then washed in water and various stains were applied. We have also used the Zenker fixation, but this was not so uniformly successful. Precipitation of the chrome and mercurial salts upon the fibrin threads gave much difficulty in later removal and treatment. Moreover, the formalin prepared material permitted the demonstration of fat deposits, which were not as well indicated in the Zenker specimens. However, for special stains the latter method was always used. In the majority of cases we applied the stain directly to the culture. It is found that much better results were obtained where the culture was made on a thin medium so that the entire mass could be stained. This avoided the subsequent cutting on the microtome. Where serial sections were made from paraffin blocks we found that great alteration of the newly developed cells took place.

The delicate protoplasm of these growing cells undergoes much shrinkage when put through the usual imbedding methods for paraffin, and the staining qualities are also considerably altered. Sudan III. and hematoxylin have formed a most useful method. Besides this, Van Gieson's connective tissue stain was useful in demonstrating fibrils of the more mature connective tissue cells. Mallory's connective tissue stain was tried in the Zenker material but gave no result on account of the fibrinous medium. Eosin-methylene blue gave fair results.

As a considerable part of the newly developing cells grows between the coagulated hanging-drop of plasma and the cover-slip, there was no difficulty in studying the stained preparations with the highest powers of the microscope. Particularly was this true when the culture was made in a very thin layer of plasma. Such a stained and prepared culture may be permanently mounted by means of Farrant's solution without exposing the section to alcohol or xylol.

As mentioned by Carrel and Burrows successful attempts at transplantation of growing cultures have been made and some importance is laid on the fact that the new growth resulting from such a transplantation is somewhat different from that of the original mass of growing cells, in that the arrangement of the cells with their processes is not as compact as in the original growth. The new cell formations have become larger in size and contain many fat globules. We have found that the actual transference of growing tissue can best be accomplished by plate culture where the growth can be readily distinguished by the naked eye and the danger of planting cells from the original mass is avoided. Best results are obtained in transplanting from two-day-old cultures while the tissues are in an actively proliferating state.

We have found that cultures once showing evidence of growth can be safely examined from time to time for about four minutes in a room at a temperature of 28° to 32° C. without endangering the life of the growth.

RESULTS OBSERVED DURING THE GROWTH OF DIFFERENT TISSUES.

Connective tissue. — This type of tissue grew most abundantly and showed evidence of growth in the cultures of all the various tissues planted. On one occasion we have observed a growth twice the size of the original mass in three hours. Connective tissue shows an earlier attempt at growth than any other type of tissue and in the average drop of plasma growth usually lasts from fifteen to twenty days. This type of cell usually assumes a spindle shape and usually a radial arrangement from the implanted mass, and as the growth extends into the surrounding fields of media the fibrillar processes as well as cell bodies and nuclei become lengthened. The arrangement of these growing cells are in the direction of the fibrin threads in the plasma, to which they cling for support. Later, as the growth extends into the media, the cells form a network causing the protoplasm of the cells to become distorted into many shapes and forms, so as to assume, in some cells, the appearance of typical nerve cells. Many cells are connected to others by means of these fibrillar processes, some having as many as six to eight limbs. After four or five days' growth we have frequently observed a few cells to wander beyond the growing area and become separated from the general mass of growing cells. These segregated cells have been found to be more round or oval in shape, their processes gradually becoming shorter and the entire cell body much larger than those cells from which they have been derived. The nuclei of these cells also assume a round or oval shape, slightly granular and very sharply outlined, while in the protoplasm of the cells many fine and a few large granules appear.

In every growing culture of connective tissue we have observed, after four days, the appearance of fine granules arising about the nuclei. Later these become larger and fewer in number, some apparently coalescing with others and extending into the fibrillar processes. These granules

are readily demonstrated to be fat globules by applying Sudan III. Such granules and globules are found only in tissues which are actually growing or at least carrying on an active metabolism in the medium. When once the culture begins to degenerate the outline of the cells becomes very indistinct, the protoplasm granular or containing vacuoles, and the nuclei lose their prominence and become fragmented.

Many cells, especially those which are embryonic in type, not infrequently have two or more nuclei, but few nuclear figures were observed.

It has been our repeated observation that mitotic figures are not as commonly present as might be expected. In examining cultures at various periods, from those only a few hours old to those which had grown for some days, we were much astonished that although there was an unquestionable increase in the number of cells in the media, only rarely were nuclear figures present. We have repeatedly observed that the nuclei of the cells showed stages simulating closely a process of amitosis, some nuclei showed bilateral indentations like the figure of eight, others were lying side by side with only a short link of nuclear material between them, while again many cells were observed with two distinct nuclei lying close to each other. It should be pointed out that in the older cultures and more particularly in the mass of the implanted cells, various nuclear changes indicating degeneration and fragmentation are found; these, however, differ much from the character of the nuclei seen in the growing cells.

The protoplasm of the young cells is clear and almost homogeneous; there are no granules save the appearance of a very fine stippling. No structure can be made out in the protoplasmic body.

As has been pointed out by Carrel and Burrows, the growing cells of connective tissues commonly have a radial arrangement. This arrangement is mainly determined by the direction of the fibrin threads along which they grow. In an active growing culture of connective tissue there is commonly found a development of a clear area like a halo

surrounding the original mass. This halo develops with the increase of the growing cells, and probably is the result of a lytic substance acting upon the formed fibrin. It is probable that a part of the ferment acting upon the fibrin is derived from the dead cells or the cells undergoing destruction in the original implanted mass. Commonly the liquefaction of the medium takes place at a later period than the production of much of the new growth. We must say, however, that in no case have we observed the development of the circumscribed destruction of fibrin in preparations showing no growth of the implanted tissue. When the area of liquefaction is well advanced, the connective tissue cells continue to grow towards the periphery, but not with the same definite arrangement as the early cells growing along the fibrin threads. When the cells reach the border of the liquefied area they tend to grow along it so as to form a new zone or "shore line," encircling the liquid medium.

The morphology of the growing connective tissue cells differs very widely from those of the adult animal. Nevertheless the character of the growing cells is fairly constant even when obtained from different animals (chick and guinea-pig). On morphological grounds alone it was often difficult to be convinced that the cells were of connective tissue origin. The early development of the cells from the implanted tissue showed mainly spindle types, having a fairly large body and ending at both extremities in fine filaments which were attached to the succeeding cell. At times, also, filaments extended from both poles to cells which lay laterally. These filaments formed definite attachments to those cells which presented the progeny or the parent. In this way the chain or chains of cells arising from a single unit could be followed. In this way, too, many cells though morphologically different from other cells in the chain could be recognized as belonging to a certain type.

Similar to the observations made by Harrison upon nerve cells, we are led to believe that the large processes and fibrils of connective tissue are formed by the drawing apart of two cells which were first joined by heavy protoplasmic

processes. Thus in the growth of new cells the progeny wander away from the parent, but still remain attached by linking fibers.

Not infrequently the cells lying more peripherally formed large syncytial masses with large filaments and several nuclei. Again others were observed with a more spherical body having one or two large protoplasmic offshoots and smaller thread-like filaments. Morphologically these resembled nerve cells but did not contain granules in their protoplasm.

In the multiplication of some cells long ribbon-like processes are produced which sometimes end in oval masses or islands of cells which are closely apposed to each other. The individual elements of these islands are more or less circular without evidence of fibril production.

In the older cells (over ten days' growth) it was possible to obtain the reaction for connective tissue fibrils by the Van Giesen stain. By this method it was found that the cell processes stained uniformly, and did not show the presence of multiple fibers. There was no evidence that the cells produced more than one fiber in each prolongation, but at the same time a single cell could produce many processes from its periphery. By the traction of several attached cells, in the same direction, a number of the fibers may become arranged in closely aggregated bundles looking like a single process. We were unable to obtain results with Mallory's stain.

As was observed above, the young growing cells are devoid of granules in their protoplasm. As, however, these cells become older, granules appear occupying the cell immediately about the nucleus. Later, similar granules are found extending into the processes and much of the cytoplasm is filled with bright refractile bodies. Similar bodies have been described by Carrel and Burrows. In the cultivation of tissues these granules indicate degenerative changes in the cell and are found to consist of fat. Beautiful preparations may be obtained by staining these tissues with Sudan and hematoxylin. That these granules indicate a process of degeneration and are dependent upon an altered condition

of the foodstuffs is shown by their early appearance in the cells close to the original mass which was inoculated. The peripheral cells are usually last to show this fatty degeneration.

An interesting question arises as to the origin of this fat. From our observations we would conclude that it is derived from the presence of invisible fatty compounds contained in the plasma. The quantity of fat which becomes visible in these cells is, however, very remarkable. A small quantity may also be derived from the destruction of some of the originally implanted cells. We have found, however, that the total fat is not obtained from this source, as in those cultures which did not grow no considerable quantity of fat became apparent, either in the medium or in the dead cells which had been introduced.

The fat in the connective tissue cells first appears in fine globules which later coalesce to form single or multiple large globules. We have never observed the accumulation of fat in these cells to simulate all characters of a true fat cell. Moreover, fat in any quantity was never observed to accumulate in dead cells, though these may sometimes show the presence of very minute Sudan staining granules.

In a few instances we have observed the presence of cells of a signet-ring character closely resembling true fat cells. These, however, were not connected with the cells of the fibrous tissue type, and of their origin we cannot definitely speak.

Transplantations are quite readily made from actively growing cultures to fresh media, and by careful preparation successive transplantations could be carried on for many generations. In the new cultures just prepared active growth took place within an hour after inoculation. The morphology of the cells and the growth is found to be similar to that of the original culture, except that the individual cell bodies appeared larger and the arrangement of the colonies was looser. Fat also appeared in these cultures after the fifth day.

Connective tissues grow fairly well in alien plasma. Upon

inoculating human plasma with embryonic chick tissues, we obtained a rapid formation of fibrin radiating in very thick and heavy layers from the original mass of tissue. Actual growth did not appear until seventy-two to eighty-four hours after inoculation, after which time the connective tissue cells grew to the extent of twice the radius of original mass in forty-eight hours, and then gradually ceased growing, becoming granular. Upon staining this growth the cells were found very indistinct, owing to the granular condition of the media which produced a cloudy appearance of the field. This cloudy condition of the media was still more marked in human plasma inoculated with guinea-pig tissue.

Liver tissue. — Cultures of liver tissue were obtained with fair success. Occasionally connective tissue fibers were the first to grow and appeared in the early hours of the culture. The growth of the cells from the parenchyma began later, usually not before forty-eight to seventy-two hours after incubation. When the growth of the liver cells once began they continued to increase and became widely scattered in the media at some distance from the original mass.

The liquefaction of the media about the growing liver tissue was commonly observed, and it was in this fluid medium that the greater number of new liver cells were to be found. In all the cultures of liver it appeared that the growth of connective tissue fibers and that of liver cells was quite independent. In no case did the liver cells arrange themselves in any particular system along the fibrous tissue.

These liver cells were of a fairly constant shape and of a uniform size. They appeared spherical and were much larger than the cells of the parenchyma which were introduced into the medium. The nucleus was usually centrally placed, but as the cells grew older the nuclei became eccentric and in the later stages of degeneration were even peripherally placed. The nuclei were fairly large and distinct. The protoplasm was clear and without granules until in the stages of degeneration globules of fat of various size began to appear. In cultures ten to twelve days old great numbers of small

and large globules appeared in the protoplasm and gave the usual reaction for fat with Sudan. In observing the development of these fat granules it appeared as if some of them coalesced to form the larger globules.

In no case did these cells show the development of fibrils or protoplasmic processes. Other investigators have claimed to have observed the development of columns of liver cells growing from the original mass of liver tissue. This we have failed to observe in our cultures, save that in some instances small projections of cells were seen jutting out from the tissue implanted, but in all probability representing some of the original tissue. These projections could not be distinguished as growing cells and the accumulation of fat granules was different from that in the observed growing cells. That one does occasionally observe liver cells lying side by side is obvious, but such an arrangement is quite different from that of a liver column.

Similar difficulties present themselves in the recognition of growing liver cells as differentiated from other types of tissue. Although the cells were morphologically in relative size, shape, and arrangement, and in the character of the fat globules present, similar to liver cells in the adult body, the actual microchemical proof of their nature was not forthcoming. We had hoped that by the introduction of hemoglobin into the culture medium we could obtain some reaction in the medium indicative of liver cells, but our results proved negative. There was no evidence that the liver cells growing as isolated units in a hemoglobin medium could make use of this material.

Kidney tissue. — Our results in the cultivation of kidney tissue have not been uniform. The character of the growth resembled to some extent the results obtained in the cultures of liver tissue. In the cases where growth was observed the development of the radially arranged connective tissue fibers preceded the evidence of proliferation from the parenchyma. The parenchymatous cells developed as individual or isolated clusters of round cells in the clear medium beyond the

implanted tissue. The individual cells were smaller than those of the liver and the protoplasm was finely granular. There was no great tendency on the part of these cells to accumulate fat, and the granules which did develop remained quite small. The individual cells showed little tendency to remain associated with others of the same kind, and we never observed any relationship in the arrangement of the parenchymatous cells with those of connective tissue origin.

Occasionally tubules were observed along the margin of the implanted kidney substance. These tubules we believe were structures which had become loosened from the edematous implanted tissues, giving an appearance of growing tubules. In no instance has a tubular formation been observed in cells quite free from the original tissues.

Spleen tissues. — Very fair results were obtained in cultures of spleen both upon a plasma and a serum medium. These cultures showed the development of great numbers of round cells varying in size. Not infrequently these cells formed masses or clumps of cells in the clear medium and in this way there were developed new foci from which active growth took place. The majority of the cells were of the size and appearance of large and small lymphocytes. The nuclei were round, sharply outlined, and stained intensely. There was no evidence of the production of fibrils, and the cells even in the older cultures showed no tendency for the accumulation of fat. In the majority of cultures from this organ there was a development of connective tissue. This tissue had the characters as previously described and showed the accumulation of fat in its protoplasm.

Muscle tissue. — In many cultures of heart muscle, cells were found to migrate into the surrounding medium from the original mass but without evidence of growth, while the supporting connective tissue grew quite abundantly. In a few instances the development of large syncytial branched cells having multiple oval and parallel arranged nuclei was observed. Such cells had the appearance of teased out

heart muscle, but the protoplasm was of a more translucent character. In the early cultures there was no evidence of granules or globules in the cells. Later a few small globules appeared in the protoplasm. The further growth of these cells was one of increasing the size of the syncytial mass or at times the development of new protoplasmic processes. No fibrils simulating those of connective tissue were observed in these syncytial structures and the tissues stained yellow with Van Giesen.

Burrows has previously obtained successful cultures from heart muscle and claims to have noted the rhythmical contraction of these newly developed cells. This has not occurred in our cultures, although the implanted portions continued to beat for some time.

In the heart muscle cells which we believe were growing there is no evidence of transverse striations. It is therefore with some doubt that we mention these cells as definite muscle tissue. With, however, the difference in the morphological characters and in the staining qualities from the other connective tissues which we had observed, we were fairly convinced of the muscle origin of these cells.

It is to be noted that not uncommonly a number of striped muscle elements migrate from the original tissues at some distance from the culture and undergo degeneration without proliferation in the medium. These cells are not to be mistaken for the growing elements of successful cultures.

Epithelial tissues. — As previously stated the surface epithelial tissues were found to grow more rapidly and abundantly in a serum agar medium. Where the growth of the epithelial tissues predominates over the growth of connective tissue arising from adherent masses of corium, the cells take a radiating arrangement from the original mass and grow in several planes. The majority of these cells present an oval appearance, some containing one, others two nuclei and have a fairly clear protoplasm. Other cells are distorted into many shapes and forms and are of various sizes, as if in a process of division. The cells lying in the periphery of

the culture are usually individual or in pairs, and contain fine fat globules, while those about the margin of the implanted mass show a great accumulation of fat in the protoplasm of the cell.

In these cultures of skin tissue we have not observed any chemotatic attraction between the connective tissues and epithelial cells. Each type of tissue appears to grow independently of the other.

In only one instance did we find that the epithelial cells took an arrangement to the growing connective tissue. In this case the connective tissues which grew abundantly formed a well developed membrane about the liquefied area of the plasma, while epithelial cells were compactly arranged upon the inner side of the stroma. There was, however, no evidence of a Malpighian layer. Care must be taken to exclude the migrating epithelial cells from the consideration of true growth. Intercellular bridges or the presence of keratinizing cells were not observed in any cultures.

Degenerative changes were evident in some of the epithelial cells in the presence of very minute fatty granules.

During active growth mitotic figures were quite evident in some of the epithelial cells.

DISCUSSION.

The cultivation of tissues outside of the body offers an excellent means for the study of the cytology of tissues, as well as of the metabolic activity of the cells. With the technic that is now established the difficulty of obtaining growth of diverse tissues upon different artificial media is no longer very great. The results of the different investigators indicate that almost every kind of tissue of warm-blooded animals can be cultivated in vitro. Nevertheless considerable work is still necessary for the development of a microchemical or other test to conclusively prove the nature of the different cell elements which are present in each culture.

In our own experiments we have found that our greatest difficulty lay in the actual determination of the nature of the

cells which had grown. Similar to the experience of Lewis and of Lambert and Hanes we found that various tissues grew quite readily in serum media, heterologous plasma, and in simple solutions, as Ringer's preparation. It is evident, however, that the best growths are to be observed in the plasma media as described by Carrel and Burrows. It is of advantage to obtain this plasma from animals without the use of an anesthetic.

When the plasma media is inoculated with a tissue for cultivation, there is a rapid formation of fibrin which radiates from the point of implantation. This fibrin is of definite value to the growing cultures, in that the cells find support and are guided in their direction of growth. This has been very evident in all the cultures and particularly where connective tissues have actively grown. One is reminded of a similar process which is to be observed in the healing of wounds.

Another interesting point which simulates conditions arising *in vivo* is the migration of cells during the time that they are actively proliferating. It is not an unusual observation to find the new cells wandering at some distance from the parent cells and setting up new centers of growth. This characteristic of wandering is to be observed in almost all tissues and quite markedly in the epithelium of the skin tissues. It is probable that a similar migration of epithelial cells occurs on the surface of large wounds and that the new islands which form are from such points of migration. In cultures one must differentiate active migration of living cells from the passive migration of various materials and cells as is observed in the penetration of agar by red blood corpuscles lying on the surface. Similar observations were made by Carrel and Burrows.

As far as we have been able to observe, the connective and spleen tissues of adult mammalia grow most easily. On the other hand, no difficulty was experienced in growing liver and epithelial tissues from embryonic chicks. In the latter, however, it was also true that the connective tissues grow most profusely in cultures of all organs.

The actual evidence of growth is best observed in the comparison of the number of cells implanted with the number of cells present after several days' incubation. Little difficulty is experienced in recognizing a culture which has not grown, for in it the tissue shows little or no change save the disintegration of the nuclei and of the cells. In the cultures which have grown the number of cells increases many fold, and these new cells which constantly extend the circumference of the tissue mass show all evidence of vitality and of carrying on individual cell metabolism. These cells, however, differ from those which constitute the cells of the body in that they show no relationship in their metabolism or functional activity with other cells. Besides this we have never observed the dependence of growth of one type of cell upon another order of cells. Thus the development of cells of a glandular or epithelial origin is not associated with a preceding or simultaneous growth of stroma, so that epithelial tissues do not simulate the arrangement and structures of the tissue from which they were derived. It is obvious that the growth of these cells is very different from that in the body and that these cells must adapt themselves to circumstances quite different to that which we appreciate as the normal. In the method of preparing artificial cultures both the quantity and quality of food supply offered to the cells is arbitrarily the same. It is impossible thus to obtain for the different cells the preformed nutritive materials which characterize their metabolism in the body.

It would appear from this that the growing cells in vitro become isolated entities like so many amebæ differing only from each other in the degree of specialization, which they have acquired prior to their growth under artificial conditions. It is this increased specialization, which continues in the developing embryo, which increasingly offers greater difficulty in obtaining cultures in vitro. The results of successful cultures of any tissue may be compared with the autochthonous growth of tumor. Whether the comparison can be carried still further is yet to be determined. In a single instance we have obtained negative results from the

implantation of actively growing embryonic tissue cultures into the homologous animal. It does, however, appear possible that the gradual adaptation of these cells with vegetative qualities by repeated transplantation on artificial media may so impress them with these qualities that independent and unlimited growth will continue when they are transplanted into an animal.

In all of our observations attention was paid to the presence or absence of function in the growing cells. Up to the present we have not been able to observe that any of the tissues carry out any of the functions which are normally allotted to them in the mature animals. Thus, although the liver and connective tissue cells show an abundance of fat globules, we could hardly indicate that this metabolism was carried out for a definite purpose by the cell. Moreover, the liver cells when grown in presence of hemoglobin did not appear to be able to make use of this. Likewise in the culture of adult tissues where the new cells were derived from specialized organs having previously acquired a specific function, the new progeny appear to exert their entire energy in vegetative activity without continuing their specialized function. It is probable that before we are able to have specific tissues functionate during artificial cultivation, we must properly modify the culture medium to serve the necessities of the cell. Others have already shown that the modification of the media by methods of dilution has a decided effect upon the rapidity of the growth.

One of the most interesting features of these observations has been the study of fat deposits in the various cells. Liver and connective tissues always accumulate fat in the greatest quantities. Fat usually appeared about the fourth day of growth and continued to accumulate during the life of the cell. The origin of this fat is not clear, for the amount that is seen in cultures of eighteen days' growth appears much larger than that which would be expected in the plasma or cells of the implanted mass. From the results that we have obtained up to the present the fat is of a semifluid nature and consists of a neutral compound.

Studies are at present in progress to determine the exact nature and origin of this substance.

[My sincerest thanks are due to Dr. Oskar Klotz for his supervision and assistance in this work, and to Dr. S. R. Haythorn for the photographs.]

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DESCRIPTION OF PLATES.

PLATE VII.

FIG. 1. — Growth of connective tissues forming layers and connecting strands.

FIG. 2. — Growth of connective tissues forming fibrils and strands.

FIG. 3. — Growth of isolated connective tissue cells showing presence of fibrils and fat globules.

PLATE VIII.

FIG. 4. — Newly developed connective tissue cells beginning to accumulate fat (six-day old culture).

FIG. 5. — Growth of skin tissues. The tissues shown are all newly developed and at some distance from the implanted mass. The single strand shown consists of connective tissue. The denser mass is composed of epithelial cells.

PLATE IX.

FIG. 6. — Skin tissues from specimen shown in Fig. 5. The relation of the growing epithelial cells to the strand of connective tissues is shown.

FIG. 7. — Liver cells loaded with fat (fourteen-day old culture).

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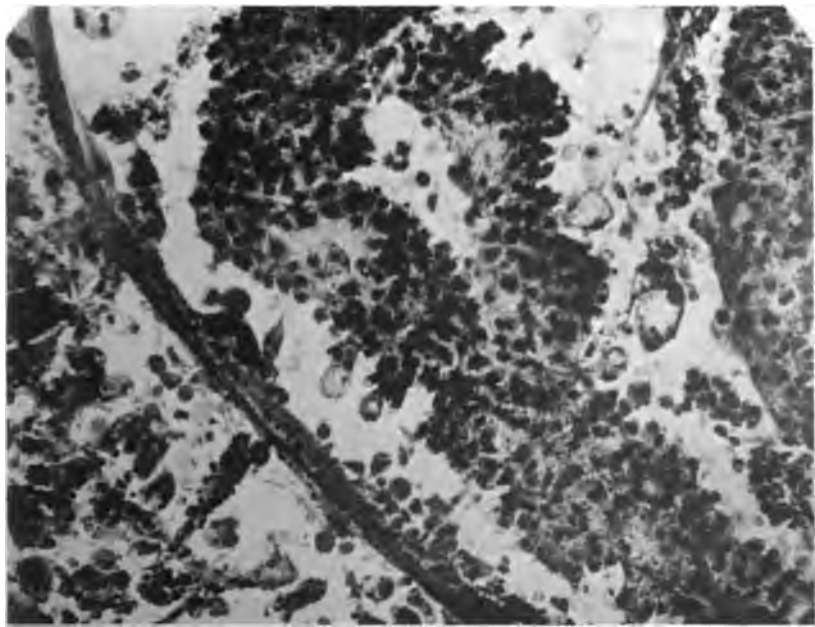
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Growth of tissue

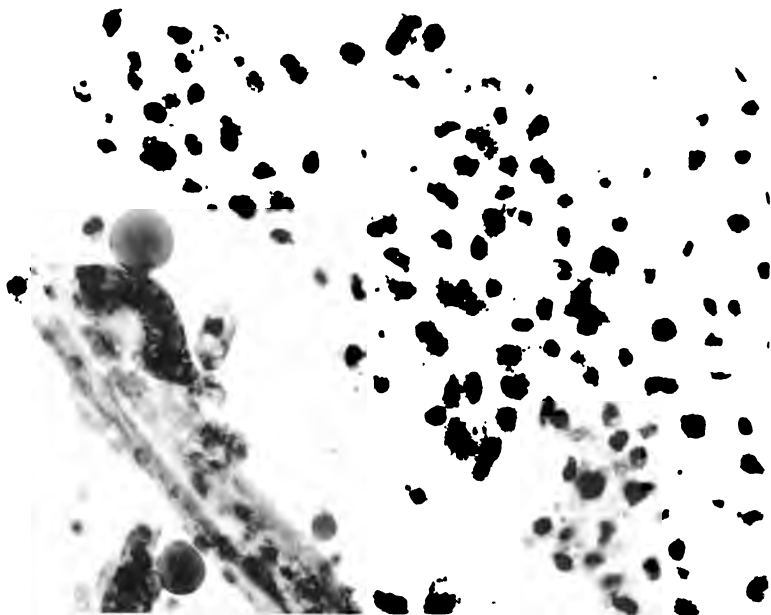


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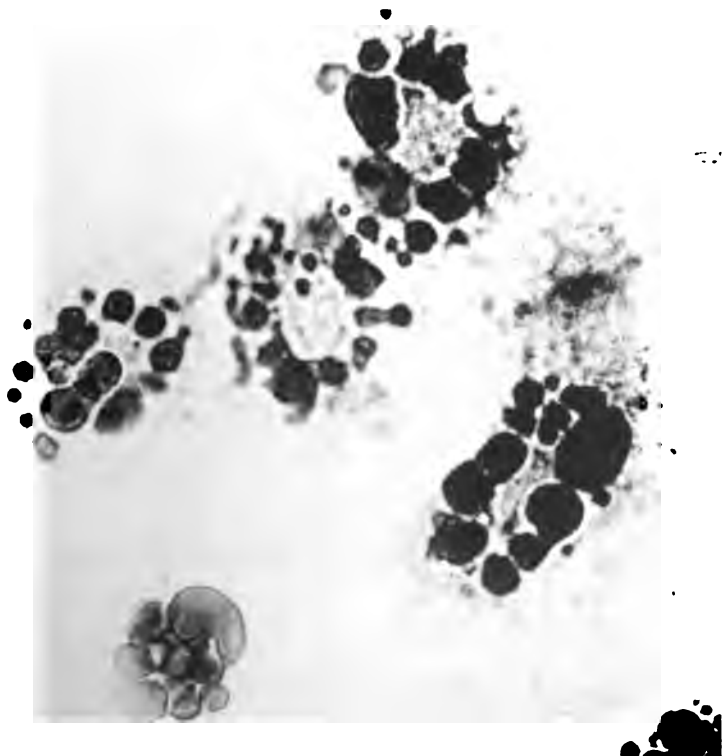


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AN EXPERIMENTAL STUDY OF MEASLES IN MONKEYS.*

WILLIAM PALMER LUCAS, M.D.

(Clinical Instructor in Pediatrics, Harvard Medical School; Physician-in-chief of Children's Department of the Boston Dispensary.)

AND

EDWARD LEWIS PRIZER, M.D., BOSTON, MASS.

(From the Department of Surgical Research, Harvard Medical School.)

The first report of the production of experimental measles dates back to 1852 when Mayr was able to prove the infectivity of the buccal and nasal secretions in man, producing measles in human subjects. In 1898 Chavigny reported the appearance of what he thought was measles in a monkey, which had been in close contact with his keeper during the early stages of an attack of measles.

Josias in 1898, stimulated by this report, allowed monkeys to play about a measles ward for a period of six months, but was never able to detect any signs of measles in the monkeys. Apparently, this negative attempt delayed further animal experimentations until 1905, when Hektoen produced measles in man by injecting blood from a human case early in the eruptive stage. In 1910 Anderson and Goldberger attempted the production of measles in monkeys by the intra-peritoneal injection of blood from a case of measles, getting what at that time they thought negative results, though upon later analysis they concluded that one of their monkeys had shown a characteristic rise in temperature on the eleventh day. Their second attempt was also apparently negative except for a rise in temperature. Their third attempt succeeded somewhat better since one of their monkeys besides the temperature rise showed signs of a sparse papular eruption on the face, brow and chin, with a diffuse erythema of the brow and lids; five days later a brownish scaling was noted. Their fourth case, however, proved beyond a doubt that the blood from a case at the end of about fourteen to twenty

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hours after the appearance of the skin eruption could produce experimental measles in the monkey. They have since carried on the virus from this case through six generations of monkeys, showing that virus can retain its pathogenicity for a considerable length of time when grown exclusively in monkeys. They were unable to grow the virus on artificial media. Their inoculations were given intravenously, intracerebrally, and intraperitoneally with about equal success. Their positive evidence of measles consisted in a more or less definite rise of temperature four to seven days after inoculation, coughing, sneezing, and the appearance of a more or less typical papular rash.

Hektoen and Eggers have recently repeated the research of Anderson and Goldberger on experimental measles in the monkey and have made a special study of the leucocytes. They conclude that "Macacus rhesus is susceptible to a mild kind of measles if injected with the virus of human measles present in the blood . . ." and that "the leucocytes appear to behave very much as they do in human measles; that is to say, that preceded by a more or less distinct leucocytosis there appears a leukopenia of variable degree in what would correspond in a general way to the latter part of the pre-eruptive and the early part of the eruptive periods." They add: "In our animals this leukopenia involved principally the neutrophils, the lymphocytes being relatively somewhat increased."

Our experiments were undertaken to study further the production of measles in the monkey, especially with reference to the blood picture changes. We obtained our blood from a case of beginning measles, about six hours before the appearance of the skin eruption and about thirty-six hours after the initial rise in temperature, at a time when the child was known to have been exposed to measles in the orthopedic ward of the Children's Hospital. Blood was withdrawn from one of the veins at the elbow under strict sterile precautions and within an hour thereafter was injected as follows into two monkeys: No. 112 received two cubic

centimeters of clear blood serum from the clot intracerebrally and three cubic centimeters intraperitoneally, and No. 113 received five cubic centimeters of serum plus blood cells obtained by vigorously shaking the tube containing the original blood specimen, two cubic centimeters of this blood cell serum suspension being injected intracerebrally and three cubic centimeters intraperitoneally.

The first signs of illness occurred in monkey No. 112 towards the end of the sixth day and consisted of listlessness, shivering, and hurried respirations unaccompanied by any appreciable rise in temperature. At the close of the tenth day both monkeys showed characteristic evidence of measles in the form of definite Koplik spots on the buccal mucous membranes. These spots in one monkey we were fortunate enough to have verified by Dr. English from the South Department of the Boston City Hospital. Late on the ninth day after inoculation and at a time when monkey No. 112 was decidedly sick we drew blood and inoculated monkey No. 115 with two cubic centimeters of the serum intracerebrally and three cubic centimeters subcutaneously. This monkey showed a similar periodicity of symptoms, Koplik spots appearing as in the first two monkeys on the tenth day. More than forty-eight hours after the appearance of the Koplik spots, monkey No. 113 was bled and from his blood serum No. 116 was inoculated intracerebrally with two cubic centimeters of the serum and subcutaneously with three cubic centimeters more. This monkey gave no certain evidence of having contracted measles.

All of the monkeys successfully inoculated showed at some time during the acute stage of the disease a transient erythema of the face and forehead but never any more characteristic eruption. The blood picture, however, taken in conjunction with the appearance of the Koplik spots and the invariable absence of both leucopenia and Koplik spots in the monkeys examined from time to time as controls proves without doubt that our monkeys were successfully inoculated with measles virus.

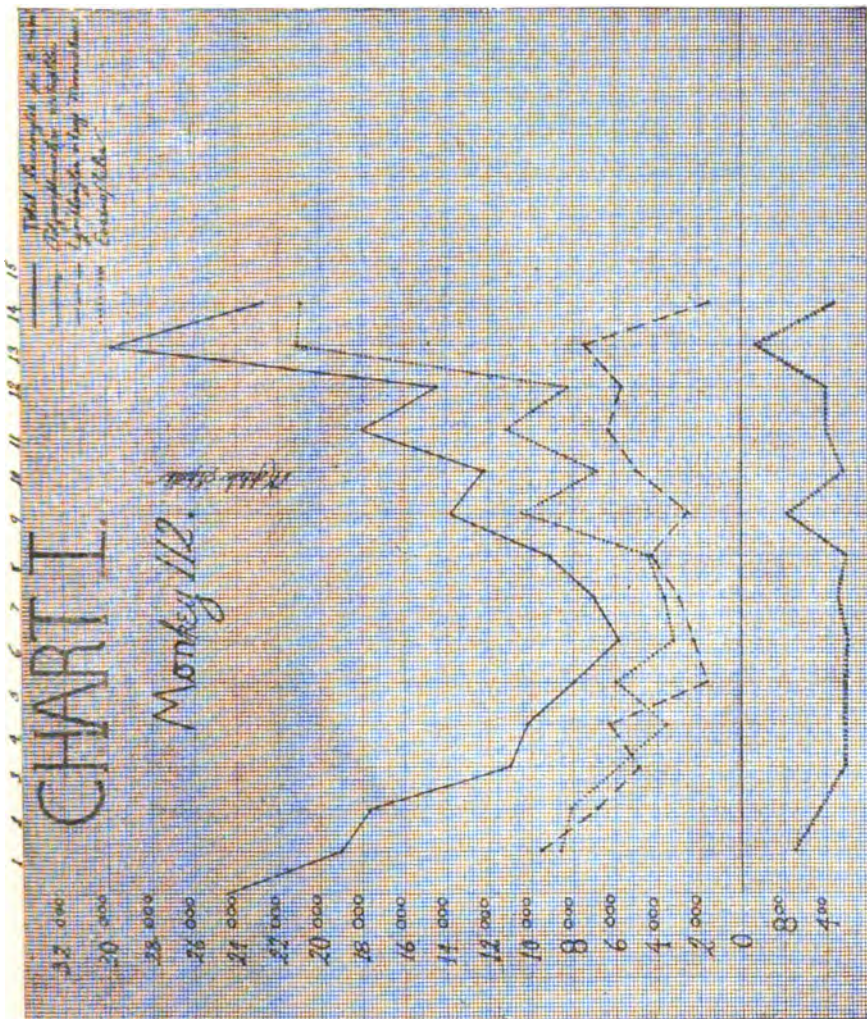
The blood used in making our counts was obtained by

puncture of the marginal vein of the ear. The leucocyte count was made immediately and cover-glass smears were prepared for further study at our convenience. Except on Saturdays and Sundays, when the hour was considerably earlier, blood was drawn each afternoon at approximately 6 P.M.

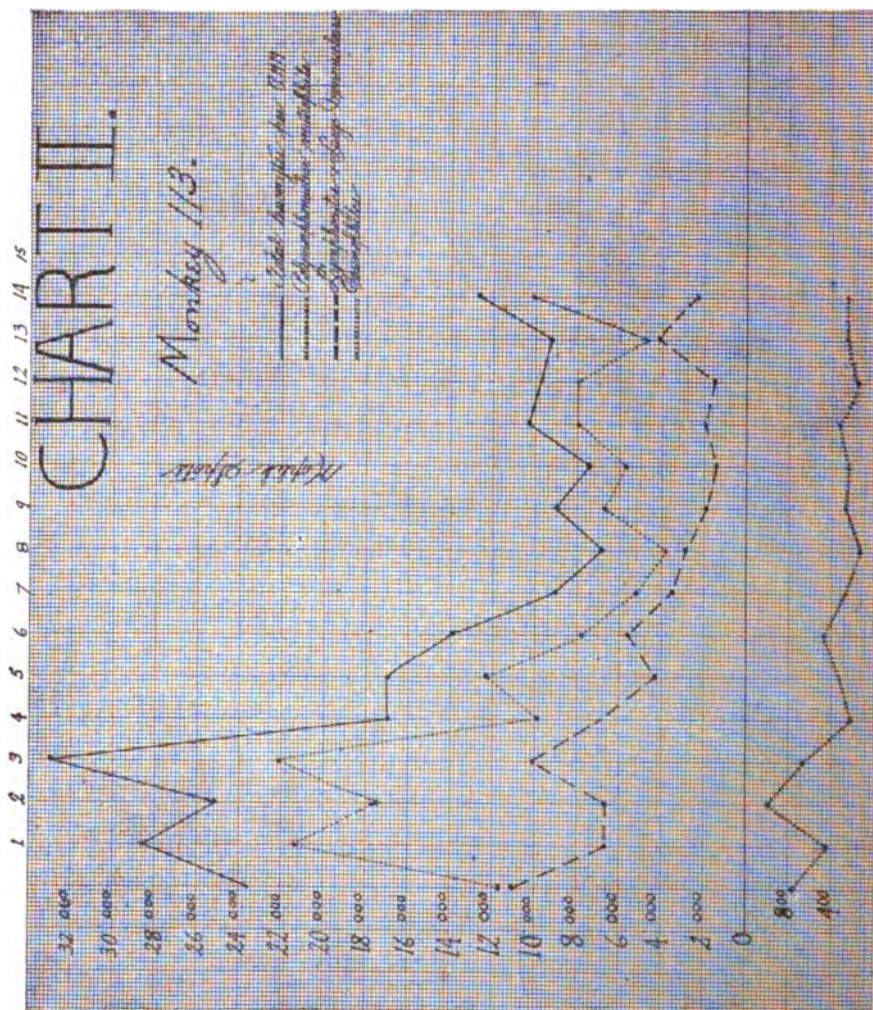
For differential counts Wright's modification of Leishmann's blood stain was used and five hundred or more cells counted. As pointed out by Hektoen and Eggers, differentiation between large lymphocytes and large mononuclear leucocytes is not always easy in the monkey's blood and we attempted to classify with large mononuclear leucocytes all those lymphocytes which exceeded in diameter twice that of a red corpuscle.

This arbitrary rule of division proved unsatisfactory, consequently in our discussion we group together large mononuclear leucocytes and lymphocytes both large and small. To simplify our results as much as possible we have reduced all percentages to number of corpuscles per cubic millimeter and to facilitate comparison we have treated in like fashion the tables given by Hektoen and Eggers from their two most typical cases. These tabulations are presented graphically in the form of charts.

Unfortunately the temperature varied so much in all our monkeys that whatever temperature reaction may have occurred is not manifest. However, in the three monkeys which we succeeded in inoculating with measles, Koplik spots were found in considerable numbers toward the close of the tenth day. We observed no maculopapular eruption, though conjunctivitis occurred in one monkey on the day following



etherization. In every instance the number of leucocytes fell rapidly, either from the first day, as in monkey No. 112, or after a transient leucocytosis, as in monkeys Nos. 113 and



115. The two charts which we have prepared from Hektoen and Eggers' tables show more strikingly than do our tables the mononuclear character of the early leucocytosis, though in one of our monkeys (No. 115) we got an identical

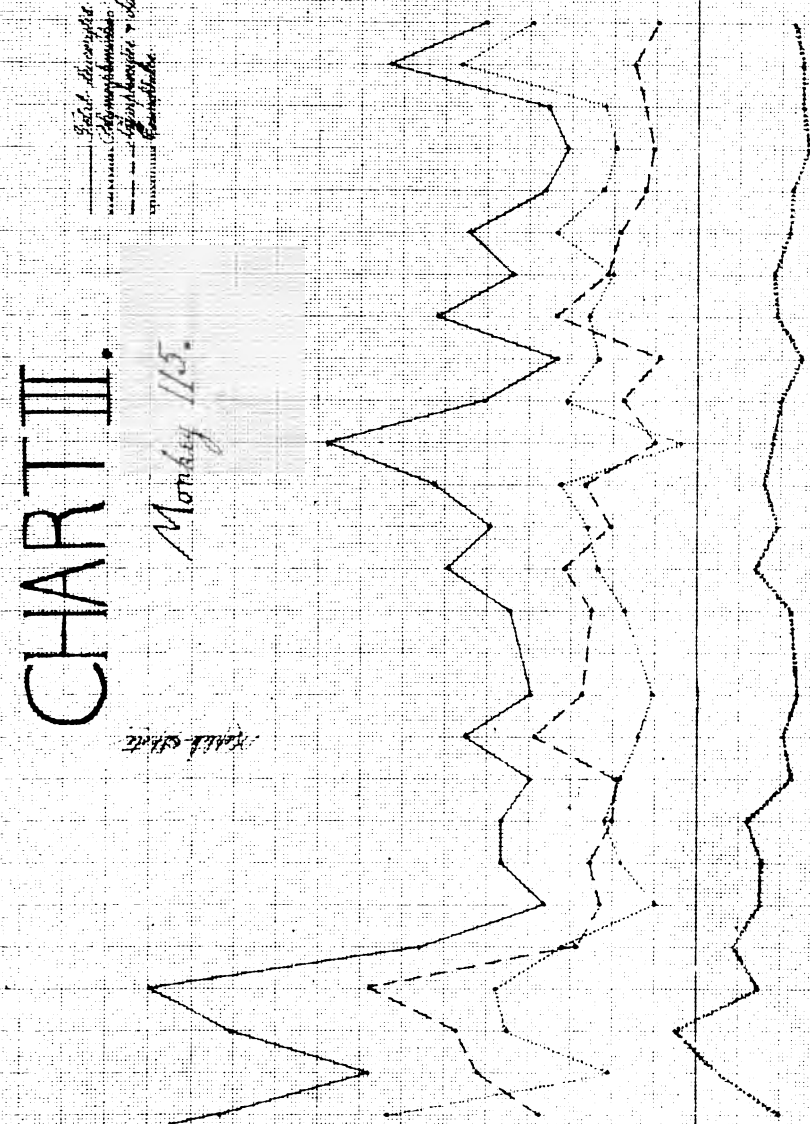
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28

CHART III.

———— *Gold Receipts for 1900*
 - - - - - *Gold Receipts for 1901*
 - - - - - *Gold Receipts for 1902*
 - - - - - *Gold Receipts for 1903*

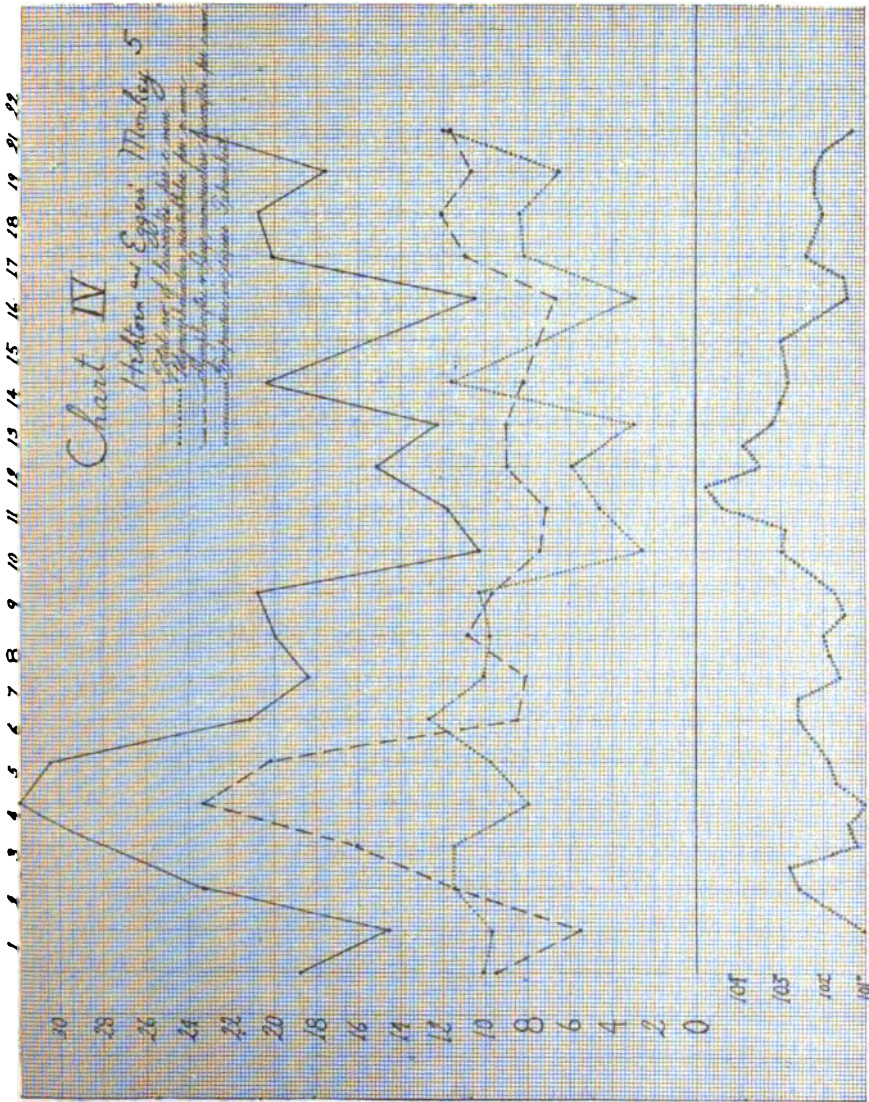
Gold Receipts
Money 115.

32,000
 30,000
 28,000
 26,000
 24,000
 22,000
 20,000
 18,000
 16,000
 14,000
 12,000
 10,000
 8,000
 6,000
 4,000
 2,000
 0
 2,000
 4,000

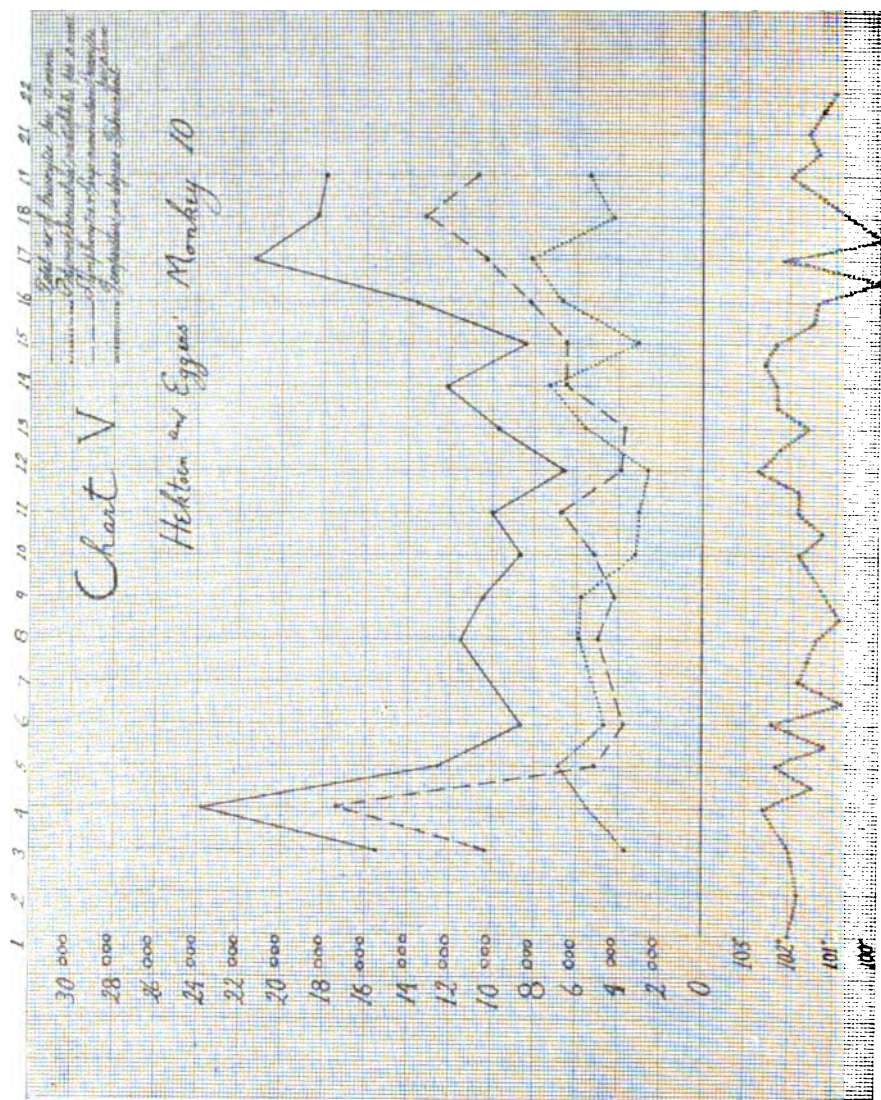


reaction. It is worthy of note that in our other two monkeys we had a slight increase in the number of lymphocytes and large mononuclear leucocytes at the end of the third day, which corresponds exactly in time with the reaction just described, although the picture is obscured in the one instance (monkey No. 113) by a marked increase in the number of polymorphonuclear neutrophils, and in the other (monkey No. 112) by a coincident fall in the total number of leucocytes.

No final explanation can be offered for these variations in the character of the reaction, but it should be noted that certain differences in technic and experimental conditions did occur. In the first place we used blood serum to inoculate monkeys Nos. 112 and 115 instead of the whole blood, and injected five instead of three cubic centimeters. In monkey No. 113, on the contrary, five cubic centimeters of unwashed corpuscles mixed with serum was the material used to carry the infection. Moreover, the blood with which Hektoen and Eggers inoculated their monkey No. 5



(Chart IV.) was drawn thirty hours after the appearance of the skin eruption, and that with which they inoculated their monkey No. 10 (Chart V.) six hours after the appearance of the eruption, while the blood with which our first two monkeys were inoculated was taken from a case of severe onset and obtained six hours before the appearance of the



skin eruption. Monkey No. 115 was inoculated with blood drawn from our monkey No. 112 on the day preceding the appearance of Koplik spots. It may well be, therefore, owing to the larger volume of material used for inoculation

and the earlier stage of the disease at which our blood was drawn, that our blood pictures represent a more severe reaction. This assumption is rendered more plausible by the fact that in monkey No. 10, which Hektoen and Eggers inoculated with blood drawn at a stage twenty-four hours earlier than was the case with their monkey No. 5, the early mononuclear leucocytosis was less marked and the leucopenia developed earlier and was more severe, so that a lower count was obtained in monkey No. 10 after six days than in monkey No. 5 after ten days.

That Hektoen and Eggers err in supposing that the leucopenia involves principally the neutrophils is shown, we believe, by a careful analysis of the five charts. To be sure the neutrophils suffer more severely in Hektoen and Eggers' monkey No. 5 (Chart IV.), as is the case in our monkey No. 115 (Chart III.), but even here the leucopenia seems to involve all the white corpuscles with the possible exception of the eosinophiles and mast cells, concerning the variations of which no conclusions can be drawn from our work. In our monkeys Nos. 112 and 113 (Charts I. and II.) the lymphocytes and large mononuclear leucocytes seem if anything relatively fewer than the neutrophils, while in Hektoen and Eggers' monkey No. 10 (Chart V.) the curve is approximately midway between the two extremes.

No red corpuscle counts were made but the smears showed evidence of but trifling anemia and this only late in the course of the disease. Once in a while a normoblast was noted but these were always rare.

The later blood pictures, which correspond with the eruptive and post-eruptive stages of the disease, are not included in our discussion since it seems probable that in all instances this part of the curve was complicated in our monkeys by an intercurrent infection the exact nature of which is unknown. After the death of our first two monkeys a number of the uninoculated monkeys became ill and several died. Only one of these had a leucocyte count under twenty thousand and in no instance were Koplik spots present. Autopsies failed to reveal the cause of death.

CONCLUSIONS.

We conclude from these experiments :

1st. That measles can be experimentally reproduced in *Macacus rhesus* and that so reproduced it is a disease of definite incubation period.

2d. That besides the fever, conjunctivitis, rhinitis, and skin eruptions described by Anderson and Goldberger it is characterized by Koplik spots (to which we believe we are the first to call attention in the monkey) and by a typical blood picture at least during the pre-eruptive stage.

3d. That the virus of measles is present in the blood serum at some time exceeding twenty-four hours before the appearance of the Koplik spots and persists until more than thirty-six hours after the appearance of the skin eruption.

4th. That during the pre-eruptive stage of the disease there is a leucopenia involving the polymorphonuclear neutrophils, the lymphocytes and the large mononuclear leucocytes. This leucopenia develops in from five to ten days after inoculation and may be preceded by a transient lymphocytic and large mononuclear leucocytosis, which is probably lacking or only poorly developed in the severe form of the reaction but is strongly developed in less severe cases.

Monkey No. 112 :

Monkey No. 112, injected with human serum as described above, showed an immediate and rapid fall in the number of leucocytes which reached their minimum at the end of the sixth day. Both polymorphonuclear neutrophils and mononuclear leucocytes (including lymphocytes) participated about equally in this reaction. On the sixth day the monkey seemed listless, chilly and respiration was hurried, but on the day following all evidences of illness had disappeared. From the sixth to the ninth day there was a slight relative increase in the number of lymphocytes and large mononuclear leucocytes. From the ninth day there was more fluctuation in the number of polymorphonuclear neutrophils than of lymphocytes and mononuclear leucocytes, the neutrophils being relatively numerous. At the end of nine days the monkey again seemed ill and ten cubic centimeters of blood were drawn under ether from the saphenous vein and used to inject monkey No. 115. On the next day Koplik spots were observed, but otherwise the animal seemed again normal. On the

fourteenth day the monkey became suddenly very ill and died before next morning. Autopsy by Dr. Boretti failed to find any cause to explain the death.

Monkey No. 113:

Monkey No. 113, injected with corpuscles and serum from the human measles blood as described, showed an initial polymorphonuclear leucocytosis followed by a leucopenia which affected polymorphonuclear neutrophils and mononuclear leucocytes (including lymphocytes) to about the same extent. The neutrophils and the total number of leucocytes both reached their minimum at the end of the eighth day, but the lymphocytes and mononuclear leucocytes continued to fall until the end of the tenth day when Koplik spots were first observed. On the twelfth day ten cubic centimeters of blood were drawn under ether from the saphenous vein and used to inject monkey No. 116. On the following day our monkey seemed sick, was listless and chilly, had a marked conjunctivitis and erythema of the face and forehead. He was much the worse on the following day and died before the next morning. Autopsy by Dr. Boretti did not find any cause to explain the death.

Monkey No. 115:

Monkey No. 115, injected with serum from monkey No. 112 as already described, showed an immediate fall in the number of leucocytes, especially the neutrophils, followed by a transient leucocytosis in which the lymphocytes and mononuclear leucocytes predominated. The total of leucocytes, the polymorphonuclear neutrophils and the number of lymphocytes and large mononuclear leucocytes all reached their minimum towards the end of the sixth day. Between the eighth and tenth days there was a slight increase in the number of lymphocytes and mononuclear leucocytes accompanied by a slight fall in the number of neutrophils. On the evening of the tenth day Koplik spots were observed in considerable numbers and persisted until the end of the eighteenth day. The blood picture after the tenth day was not significant. The neutrophil leucocytosis occurring on the seventeenth day was probably due to slight sepsis of one ear. Death occurred on the twenty-eighth day. Autopsy failed to reveal the cause of death.

Monkey No. 116:

Monkey No. 116, injected with serum from monkey No. 113, died on the eighth day. The blood picture was in no way significant. There was an initial transient leucocytosis followed by a gradual leucopenia, the decline beginning with the third day and the lymphocytes and mononuclear leucocytes falling more rapidly than the polymorphonuclear neutrophils. The lowest leucocyte count obtained was 11,000 three hours before death. There is no conclusive evidence that this monkey contracted measles.

Cause of death is unknown.

[We wish to thank Dr. E. H. Bradford for permission to work in his ward and to express our deep indebtedness to Dr. M. J. English for examining one of our monkeys and verifying the appearance of Koplik spots.

To Dr. A. F. Boretti we are indebted for complete autopsies on two monkeys.]

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HAROLD C. ERNST, M.D.,
Editor of The Journal of Medical Research,
240 Longwood Avenue,
Boston, Massachusetts, U.S.A.

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PARASITIC PROTOZOA FROM THE GAMBIA.*

Second Report of the Expedition of the Liverpool School of Tropical Medicine to the Gambia, 1911.

JOHN L. TODD, M.D.

(Associate Professor of Parasitology, McGill University.)

AND

S. B. WOLBACH, M.D.

(Assistant Professor of Bacteriology, Harvard University Medical School.)

The main work of an expedition sent to the Colony of the Gambia at the beginning of 1911 by the Liverpool School of Tropical Medicine was to observe the amount and distribution of human trypanosomiasis existing there, and to determine the best method of recognizing persons suffering from that disease.¹ As opportunities occurred, observations were made on other questions, but these were always subordinated to the main work of the expedition. Consequently this paper, which presents the results obtained by the examination of a small number of Gambian animals for parasitic protozoa, is merely the record of a series of hurried examinations made when leisure and opportunity permitted.

Practically all of the animals and birds examined had been shot and were dead when they were brought to us. In order to lessen the chance of contamination, the blood examined was always taken from the heart as soon as possible after death with a capillary pipette. But the wounds which killed the animal frequently injured the alimentary canal, and sometimes one or more hours intervened between the shooting and the making of the preparation. It is therefore

* Received for publication Feb. 15, 1912.

probable that in some instances the parasites seen in the blood found their way into the circulation after the animal was wounded. This is especially true of birds and other animals which were killed with small shot. For example, the presence of the trichomonas found in the blood of a bush fowl, and the flagellate parasites seen in the blood of a roan antelope which was shot several times, are probably to be explained in this way. When large animals were killed, the vessels of the heart were ligated, and the organ, with the blood contained in it, was brought to camp for examination.

The usual procedure followed was to examine fresh cover-slip preparations of the blood with a magnification of about four hundred and twenty diameters; as a rule, stained preparations were not made unless something of interest had been seen in the fresh preparations. The blood smears to be stained were allowed to dry; they were then fixed in alcohol and stained by Giemsa's method. The stained preparations were preserved and they were examined some months after the expedition had left Africa. Such methods are not ideal ones for a study of the morphology of parasitic protozoa; but they are the only ones which can be conveniently employed by those situated as we were. Similar methods were followed on previous expeditions to the Gambia and to the Congo. Reports were published to describe the parasitic protozoa observed during those expeditions;^{2,3} the present paper will supplement them. For convenience of comparison, the same plan is followed in the composition of all three reports.

The following is a list of the animals, giving the numbers of each examined:

MAMMALS:

Man	362
Baboon (<i>Cynocephalus</i> sp.)	2
Monkey (Gray — <i>Cercopithecus</i> sp.)	1
“ (Red — <i>Cercopithecus rubrus</i>)	1
Horse	7
Cattle	1
Antelope (<i>Hippotragus equinus</i>)	4
“ (<i>Cervicapra arundinum</i>)	7

Dog	2
Goat	1
Rats (<i>Epimys rattus</i>)	9
“ (<i>Epimys</i> sp. juv.)	1
Squirrels (<i>Heliosciurus punctatus</i>)	3

401

BIRDS:

Pelican (<i>Pelecanus onocrotalus</i>)	1
Plover (<i>Cursorius</i> Sp.)	1
Bush turkey (<i>Lisotis melanogaster</i>)	1
Crown bird (<i>Balearica pavonina</i>)	2
Sand grouse (<i>Pterocles</i> sp.)	3
Marabout stork (<i>Leptoptilus argala</i>)	3
Pigeons (<i>Vinago nudirostris</i>)	3
Doves (<i>Turtur senegalensis</i>) }	18
“ (<i>Streptopella vinacea</i>) }	
Guinea fowl (<i>Numida meleagris</i>)	2
Bush fowl (<i>Francolinus</i> sp.)	23
Stone pheasant (<i>Ptilopachus fuscus</i>)	3
Vulture (<i>Neophron monachus</i>)	2
Hawk (<i>Milvus ægyptius</i>)	8
Hawk (<i>Dissodectes ardesiacus</i>)	2
Hawk “Large gray hawk” (<i>Melhierax</i> sp.)	2
Shrike (<i>Corvinella corvina</i>)	2
Kite ()	1
Parrots “green” ()	1
Parrakeet (<i>Turacus</i> sp.)	1
Blue rollers (<i>Coracias abyssinicus</i>)	7
“Black fly catcher” (<i>Dicrurus atripennis</i>)	1
Great horn bill (<i>Bucorax abyssinicus</i>)	2
Hornbill (<i>Lophoceros nasatus</i>)	1
Babler (<i>Crateropus platycercus</i>)	1
Scapular crow (<i>Corvus scapulatus</i>)	2
Ox picker (<i>Buphaga africana</i>)	1
Oriole (<i>Oriolus auratus</i>)	1
Gray plantain eater ()	1
Jay ()	1
Weaver finch (<i>Estrela minima</i>)	7
Unidentified birds (5 species)	4

108

REPTILES:

Lizards (<i>Agama colonorum</i>)	19
“ (<i>Lygosoma</i> sp.)	4
Chameleons (<i>Chameleon gracilis</i>)	2
Snake (Viper “Puff Adder”)	1
Monitor (<i>Varanus niloticus</i>)	1
Tortoise (<i>Sternotherus derbianus</i>)	1

28

MAMMALS:

MAN: Trypanosomes.

During the course of the expedition, trypanosomes were found in seventy-nine persons by the examination of blood or of gland juice.¹ No other parasitic protozoa beside malaria were seen in the natives examined, although the possibility of meeting with spirochetes or with herpetomonads (*Leishmania*) was always remembered.

Hæmosporidia.

The tertian, quartan, and æstivo-autumnal types of malarial parasites were all seen. Of these, the tertian was seen most often in the three hundred and twenty stained preparations of blood which were made from natives of all sorts in different parts of the Gambia. In the accompanying table, drawn up to show the incidence of malarial infection, the persons examined are classified according to their age.

Age in Years	Children.			Adults.			Totals.
	0-5	5-10	Total.	10-15	15-+	Total.	
Malaria.....	5	36	41	30	8	38	79
No malaria.....	3	72	75	77	89	166	241
Total	8	108	116	107	97	204	320
	Percentage of natives under 10 infected, or "Malarial Index," 35.3%			Percentage over 10 infected, 18.6%.			

The percentage of children infected is less than it was in 1901⁴ and in 1902 (unpublished observation); but it is not fair to compare the figures because the observations were not made at exactly the same time of the year, nor on the same population.

CATTLE AND HORSES: Trypanosomes.

Trypanosomes were found in large numbers in the blood of an imported Ayrshire bull; the infection had probably existed for some time and the bull, though still on its legs, was almost moribund when it was seen. The trypanosomes in the stained preparations made of its blood are of two distinct types: one has a free flagellum, the other has not. The trypanosomes with free flagella measure from 20 to 25 microns in length; the measurements of a typical one are: I., .5 micron; II., 8 microns; III., 2 microns; IV., 8 microns; V., 5 microns; VI., 1 micron; total length, 23.5 microns.* In these trypanosomes, the kinetonucleus often lies at the extreme end of the parasite. The smallest of the trypanosomes without free flagella are typical "tadpole forms;" a few larger ones occur, and they resemble "stumpy forms." An aflagellate trypanosome of average size measures from 8 to 11 microns in length, and from 1 to 1.5 microns in width; in these forms also the kinetonucleus is often terminal.

Trypanosomes were also found in the blood of the native horses used during the expedition; both flagellate and non-flagellate forms were found in them. Rats were inoculated from the horses, and they, with one of the horses, were sent to the Runcorn Research Laboratories of the Liverpool School where the trypanosomes with which they are infected will be studied.

The form of these trypanosomes resembles that of the parasites found in the Gambia⁵ and described as *Trypanosoma dimorphon*. It is impossible to say more than this concerning the identification of these parasites from our examination of their morphology.

* The Roman numerals, by which the measurements of the trypanosomes are indicated, refer to those measurements which are taken in recording the dimensions of trypanosomes.³ They are: I., Posterior extremity to center of kinetonucleus; II., Center of kinetonucleus to posterior border of nucleus; III., Posterior to anterior border of nucleus; IV., Anterior border of nucleus to anterior extremity of cytoplasm; V., Length of free flagellum; VI., Greatest body width, without undulating membrane — usually taken opposite the nucleus.

ANTELOPE (*Hippotragus equinus*): Spirochetes.

A few spirochetes were seen in the preparations of stained blood. They vary considerably in appearance and consequently they may be of more than one species.

The commonest ones (Figs. 3, 4) are thick and measure about 5 to 7.5 microns in length. They taper slightly towards each end and end bluntly. Their protoplasm is usually homogeneous; a few of them contain a small, clear area. They usually lie in gentle waves; but they may form three or four complete waves. A few small spirochetes resembling these occur. They may measure as little as three microns in length and they are only slightly curved.

Spirochetes of another type are very slender, much waved organisms with very slender tapering ends (Fig. 5). Their protoplasm is homogeneous and they measure about five microns in length. The measurements of these spirochetes are only approximately accurate, since the smaller curves of the parasites were not accurately followed in making them.

All of the parasites described under this heading were found in blood taken from a half grown roan antelope, killed at Dungu, a town near the mouth of the Gambia River.

Trypanosomes.

A few trypanosomes were present; most of them are "tadpole forms," which measure about thirteen microns in length (Fig. 1). There is also a broader form (Fig. 2) measuring 15.6 microns in length by 3.7 microns in breadth. Two white rats which were inoculated with blood containing these trypanosomes, alive, have never become infected. It is unfortunate that this trypanosome could not be brought living to England to be studied. Its morphology does not differ greatly from the trypanosomes found in cattle and horses. Consequently, it is not impossible that the trypanosomes seen in cattle and horses and in this antelope may all have been *Trypanosoma dimorphon*.

Piroplasmata.

Red cells infected by piroplasmata occur in almost every field of the blood smear made from this antelope. Most of the parasites are rounded (Fig. 6) and contain a single mass of chromatin. This mass is usually crescentic in shape (Figs. 6-11), but it is sometimes divided into two distinct parts (Figs. 7, 13). Elongated bacillary forms (Fig. 9, 10) are not uncommon. Double infections of the same cell (Fig. 11) are very rare, as are also bigeminal forms of the parasite (Fig. 12).

Cross-shaped divisional forms were not seen. The parasite is a small one (Fig. 8); a round form of average size measures about one micron in length and a bacillary form from 2 to 2.5 microns. The red cells of this antelope measure about 4.5 microns in diameter.

Granules resembling chromatin (Fig. 13) sometimes lie within or at the periphery of the red cells. Their nature is not known, but Theiler's description of *Anaplasma marginale*⁸ makes it necessary to mention them.

This piroplasm seems to be most nearly related to those which have been called *Theileria* in the classification of the piroplasms proposed by Franca.⁷ It differs considerably from a piroplasm found in the blood of an antelope of another species.⁶ Consequently, in the event of this being a new species, the name *Theileria hippotrangi* is proposed for it.

There are many cattle in the Colony of the Gambia. There were none in the forest where this antelope was shot, but there were considerable herds at no great distance from it. Although each chief was questioned concerning it none had heard of red-water in cattle and we found no piroplasmata in the blood of the cattle examined by us in the Gambia in 1902 and in 1911.

Miscellaneous.

Flagellated (Figs. 14, 15, 16) and spore-producing, encysted (Figs. 17, 18, 19) organisms are also present in the stained preparations of this antelope's blood. These preparations were made with blood taken by a pipette from

the excised and ligated heart; but the animal had received several wounds and the preparations were not made until two or three hours after it was killed. Consequently it is very possible that the bodies illustrated in Figs. 14-19 are the result of contamination, probably from the alimentary canal.

RATS: Trypanosomes.

Trypanosomes identical in appearance with *Trypanosoma lewisi* were found in the blood of rats (in *Epimys rattus* and in an immature rat of the same genus but probable different species) caught at several widely separated localities.

BIRDS:

VULTURE (*Neophron monachus*): Leucocytozoan.

A parasite exists in white cells of a vulture shot at Sokuta which much resembles those occurring in the blood of dogs and small rodents, and named *Leucocytogregarina* (Porter⁹). It occurs in fair numbers. It measures about 7.5 microns in length by 3.75 microns in breadth (Figs. 20-25). As a rule its cytoplasm is rather coarsely alveolar and stains a deep blue, but in some parasites the cytoplasm is homogeneous and stains faintly. At one end of the parasite there is often a pale area like a vacuole which may occupy its whole width. The nucleus is usually a rather loose mass of granular material which lies near the center of the parasite and it may extend completely across it. Sometimes the nucleus is smaller and more compact or, rarely, its chromatin may be arranged in masses placed at its periphery or in lines radiating from its center (Fig. 24). Rarely, also, the nucleus may be placed at one end of the parasite. Occasionally there are one or two chromatophilic granules lying in the cytoplasm outside of the nucleus. In some specimens where a vacuole lies near the surface it can be seen that the "skin" of the parasite stains pink.

Free parasites are not seen; all are intracellular. They are usually within large mononuclear cells, but they do occur within eosinophilic (Fig. 24) and neutrophilic leucocytes (Fig. 25). The parasite is almost invariably closely connected with the nucleus, and very often the nucleus seems to

have been split by it (Fig. 24). There is usually a sharply-defined, clear area between the parasite and the cytoplasm of the host cell; in some instances there are appearances which suggest that this clear area represents a cyst envelope. Most of the host cells seem to be normal; some of them are obviously degenerated and their cytoplasm and nuclei are enlarged and faintly staining.

Parasites of this type have been found in a considerable number of small mammals. An excellent review of them has recently been published by Franca and Pinto.¹⁰ This is the first time that a parasite of this description has been seen in the blood of a bird. Should it prove to be a new species the name *Leucocytozregarina neophrontis* is proposed for it. Because one parasite of this type is acquired by its vertebrate host through the ingestion of ectoparasites (mites¹¹) containing them, the occurrence of a leucocytozregarina in a foul-feeding vulture is suggestive, when considered in association with this manner of transmission.

White cell parasites were seen in none of the mammals examined. They were sought for because free hemogregarine-like parasites had been seen in the blood of Gambian dogs in 1903.³

HORNBILL (*Lophoceros nasatus*): Trypanosomes.

Trypanosomes of two distinct types, one large, the other small, are present in the blood of a hornbill.

The large trypanosome (Fig. 26) has darkly staining cytoplasm, and it is striated from end to end by about eight definite longitudinal lines or myonemes. The nucleus stains faintly, and its details are obscured by the myonemes. It is separated from the cytoplasm by an unstained area. The kinetonucleus is large, compact, and deeply staining; it is placed midway between the posterior extremity of the parasite and the nucleus. Immediately anterior to it lies a very definite, clear, vacuole-like area. The undulating membrane is ample and its thickened edge, which arises from the kinetonucleus, is prolonged in a free flagellum. The free flagellum is probably longer than it is represented to be in

Fig. 26. The measurements of this trypanosome are: I., 14 microns; II., 5 microns; III., 3.5 microns; IV., 15 microns; V., 4.3 microns; VI., 5 microns; total length, 41.8 microns.

The small trypanosome (Fig. 27) has an elongated and very slender posterior extremity. Its cytoplasm is granular, and it is striated throughout its length by longitudinal myonemes; the striations are not so conspicuous as they are in the large trypanosome. The nucleus stains well, and it covers three-quarters of the breadth of the parasite, and lies within a clear area in the cytoplasm. The kinetonucleus is large, very dense, and very deeply staining. It is always placed at about the center of the curious prolongation of the posterior extremity. As a rule this prolongation stains pink; consequently it probably consists of the periplast alone and the cytoplasm is not continued into it. The measurements of this trypanosome are: I., 2.7 microns; II., 8 microns; III., 1.6 microns; IV., 7 microns; V., 7 microns; VI., 4 microns; total length, 26.3 microns.

Trypanosomes dissimilar to these have been seen in hornbills.^{3, 12} There is some resemblance in the prolongation of the posterior extremity between these trypanosomes and those seen in a bee-eater¹² and in a guinea-fowl.¹³ A trypanosome with a tapering posterior extremity has also been seen in a bush fowl.³

DOVES (*Streptopella vinacea*): Trypanosomes.

Trypanosomes (Fig. 28) were found in the blood of two doves shot at Somita. The cytoplasm of these parasites stains deeply, but it is coarsely alveolar, and it contains many irregular, lighter-staining areas. Eight longitudinal striations run the whole length of the trypanosome. The nucleus stains lightly. It is placed near the center of the parasite and occupies almost three-quarters of its breadth. The kinetonucleus is very compact and stains deeply; it is sometimes distinctly oblong in shape. It may lie a third nearer to the nucleus than it does in Fig. 28. The undulating membrane is scanty and its thickened edge is prolonged in a short, free flagellum. The measurements of this

trypanosome are: I., 14 microns; II., 4.8 microns; III., 3.6 microns; IV., 16 microns; V., 5 microns; VI., 5.2 microns; total length, 43.4 microns.

BUSH FOWL (*Fringilla sp.*): Trypanosomes.

There are trypanosomes in the blood smears from one of the bush fowl in which the leucocytozoön described below was found. There are two types of trypanosomes: one has a sharp posterior extremity (Fig. 29), the other has a blunt one (Figs. 30 and 31); otherwise they are very similar in appearance and though they are rather larger they resemble the trypanosomes seen in the blood of a hawk in which leucocytozoa were found.³ They also somewhat resemble the trypanosomes seen by Mayer¹⁴ in the blood of birds harboring leucocytozoa.

The trypanosomes with sharp posterior extremities (Fig. 29) stain more deeply than do those with blunt ends (Figs. 30 and 31). The cytoplasm of both is loosely granular and contains more or less numerous pale areas which have the appearance of definite but irregular non-staining granules; both have eight distinct longitudinal striations. The kinetonucleus is very compact and stains deeply. The nucleus stains freely and is loose in texture. The undulating membrane is ample, and its thickened edge is prolonged in a free flagellum. The trypanosomes with blunt ends have the following measurements: I., 3.3 microns; II., 14.5 microns; III., 3.3 microns; IV., 27 microns; V., 8.5 microns; VI., 7.5 microns; total length, 56.6 microns. The measurements of those with sharp ends are: I., 10 microns; II., 15 microns; III., 3.7 microns; IV., 25.6 microns; V., 6.4 microns; VI., 5.5 microns; total length, 60.7 microns.

Trichomonads.

The trichomonads (Fig. 32) found in a stained preparation of blood from a bush fowl probably found its way into the circulation from the alimentary canal by way of the shot wounds which killed the bird.

Leucocytozoan.

Leucocytozoa (Figs. 33-42) were found in each of four bush fowl which were shot at Tuba; this is the only occasion

on which parasites resembling *Leucocytozoan ziemanni* were seen.

The leucocytozoa were not noticed until the stained preparations of blood were examined. Most of the parasites are large, "adult" forms (Figs. 33, 34, 35, 36); the dark staining female parasites (Figs. 33, 34) are from four to five times more numerous than the lighter colored males (Figs. 35, 36). Small "young" forms are found in the preparation from each bird, but they are very rare.

The adult leucocytozoa vary in size. They may measure from 13 by 15 microns to 7 by 24 microns; they usually measure from 19 to 21 microns in length and from 6 to 7 microns in width. There is no constant difference in size between the males and females. The two smallest parasites seen measure, respectively, 5 by 2.5 microns, and 5 by 5 microns.

The commonest form of the parasites, with its host cell, constitutes a spindle-shaped body (Figs. 33-36) which may measure from 30 to 50 microns in length and from 6 to 13 microns in breadth. This body contains the cytoplasm and nucleus of the host cell and the cytoplasm and "nuclear bodies" of the parasite. The cytoplasm of the host cell is structureless and stains pink or lilac; it forms a sheath for the parasite and its extremities may be blunt (Fig. 42) or tapering (Fig. 36). The cytoplasm of the host cell has been described as part of the parasite and has been called the "periplast."⁸ The observations recorded by those who have examined many parasites in fresh and stained preparations show this description to be a mistaken one.¹³ The nucleus of the host cell may be much distorted. It is usually placed peripherally near the middle of the parasite, and may underlie or overlie it.

The nature of the host cell containing these parasites is uncertain. Some maintain that it is a leucocyte; others that it is an erythroblast. None of the parasites described in this paper lie in cells containing hemoglobin, nor are they in large, mononuclear leucocytes. From our material it is impossible to determine definitely the character of the host cells. The type of their nuclei is that of the nuclei of the

small mononuclear leucocytes of the blood, but such nuclei might also occur in erythroblastic myelocytes.

Adult female parasites (Figs. 33, 34, 42) have cytoplasm (endoplasm³) which stains a deep blue; its texture is coarsely alveolar and it contains vacuoles so constantly that these cannot be considered to be artefacts. The bodies lying within the female parasites, which stain like chromatin (nuclear bodies³), consist of two large masses and of an indefinite number of small round granules. The larger of the two chromatophilic masses (nucleus) is an oval or irregular collection of granules which measures from 2 by 4.5 microns to 3.5 by 4.9 microns. The smaller mass (nucleolus,¹⁵ blepharoplast,³ or karyosome¹⁸) is more compact and stains more deeply than does the nucleus; it measures about one micron in diameter. These two masses are often superimposed, but they may lie side by side or be widely separated. When they lie together they are often surrounded by a round or oval pink-staining area; when they are separated this area is associated with the smaller mass. When chromatophilic granules exist they are distributed irregularly, singly or in small groups throughout the parasite.

The line,³ which was described as one of the nuclear bodies of a leucocytozoön seen in the blood of an African hawk, is not present in these preparations. Other observers have also failed to find it in the leucocytozoa which they have studied; consequently, the description of it has been adversely criticised. That description is based upon a careful examination of four stained preparations of very heavily infected blood; the statements made in it, concerning the line, are based on the study of many parasites; drawings were made of one hundred and thirty and these are recorded in sketch notes. The preparations have been recently reexamined and it is still certain that the line is not an artefact as has been suggested. We repeat: "The course of development described may be mistaken, but the descriptions . . . are accurate . . . so far as the defects of the method of preparation permit."³

Adult male parasites (Figs. 35, 36) have cytoplasm which is finer in texture and stains less densely than does that of the female parasites. They stain in bluish pink or rose, and these colors may be distributed irregularly throughout their cytoplasm. The larger nuclear body or nucleus is less compact and larger than it is in the adult female parasites; in a small proportion of perfectly preserved individuals the nucleus occupies the whole width (6 to 7 microns) of the leucocytozoön. The smaller nuclear mass is seen less frequently in male than in female parasites, but its appearance and relations are the same in both forms. The chromatophilic granules may be scattered throughout the cytoplasm and they are usually rather more numerous than they are in female parasites.

The adult male and female leucocytozoön are often bordered by a red line; it is most marked at the extremities, though it may extend completely around the parasite (Figs. 33, 34, 36).

Small forms (Figs. 37, 38, 39, 40) are much less numerous than are the adult parasites. They have a blue-staining cytoplasm and one or more nuclear bodies; the latter are a diffuse mass and one or two smaller denser bodies or granules. The amount of chromatophilic material contained in the small parasites varies; the cytoplasm of those which have most stains less deeply. They seem to have commenced to differentiate into male and female forms (Figs. 38, 39). Rounded parasites with male or female characteristics may vary in width from the smallest parasite seen, measuring five microns in diameter, up to forms of twice that size; it is probable that adult parasites may develop directly from these small intracellular forms.^{3, 16} None of the extremely small forms, which have been seen in other birds infected with leucocytozoa, were seen in these preparations. Large, rounded, adult female parasites exist which are quite distinct from the small round forms. These large forms are freed from the cytoplasm and often also from the nucleus of the host cell. Their cytoplasm stains a much deeper blue, and is more alveolar than the cytoplasm of the

small, intracellular parasites. Their rounded shape is sometimes produced by a folding inwards and overlapping of their extremities (Fig. 41). Keysselitz and Mayer¹⁶ have also seen forms similar to these.

Leucocytozoa have been seen in African francolins from Uganda and the Sudan.¹⁸ The characteristics of the parasite described here are not distinctive enough to distinguish it from those already described.

Hemosporidia.

Halteridium-like parasites were found in the blood of pigeons (*Vinago nudirostris*); doves (*Streptopella vinacea*), (*Turtur senegalensis*); guinea-fowl (*Numida meleagris*); bush turkey (*Lissotis melanogaster*); stone pheasant (*Ptilopachus fuscus*); bush fowl (*Francolinus* sp.); crown bird (*Balearica pavonina*); and vulture (*Neophron monachus*). As a rule there were very few parasites in the blood, and heavy infections were seen only in a pigeon and in a stone bush fowl and in a vulture.

Various authors (Wenyon,¹⁸ Minchin¹⁹) have suggested that there may be more than one species of halteridium. An inspection of the parasites present in these birds suggests strongly that this may be so. The adult forms of the parasite of the pigeon are large organisms, which displace the nucleus of the cell containing them; infected cells usually lose their shape and become rounded (Figs. 67, 68, 69, and 70). The adult parasites all contain a good many very coarse, dark pigment granules, and they show their male or female characteristics distinctly. The female forms are by far the most numerous; their cytoplasm is very dark and very alveolar (Fig. 69). The male forms are easily recognized by their paler cytoplasm and considerable amount of nuclear material (Fig. 70). Although the pigeon was heavily infected, there were very few young parasites (Figs. 67 and 68); almost all were mature gametocytes. Coarse pigment granules occurred in young parasites scarcely quarter grown.

Halteridia quite similar to this one were seen in two species of doves (Figs. 57, 58, and 59), guinea-fowl, bush turkey,

and crown bird. Quite different was the pigmented red cell parasite seen in the blood of a stone pheasant (Figs. 60, 61, 62, 63, and 64). The bird was heavily infected but the parasites were practically all small ones. The smallest ring forms (Fig. 60) were little more than half the width of a red cell nucleus in diameter. The parasites were irregular in shape; some of them were almost divided into two or more lobes (Fig. 62). All of them had a considerable amount of chromatin, often divided into several granules. Pigment seems to appear early; in the adult parasite it occurs in coarse granules. Infected cells are not distorted, nor are their nuclei displaced.

Somewhat similar parasites (Figs. 71, 72, 73, 74, and 75) to those from the stone pheasant were found in two francolin bush fowls, which also contained the leucocytozoön described above. Pigment in small amount is present in small, immature forms (Fig. 74), and is abundant in the larger, possibly mature, forms (Fig. 75). The division of the parasite into lobes (Fig. 73) is even more marked than in the parasite from the "stone bush fowl." The parasite illustrated in Fig. 73 is the largest one we found.

In the vulture's blood there is a halteridium of the classical type (Figs. 65 and 66). The host cells are not distorted, nor are their nuclei displaced; the adult parasite extends about them in the shape of old-fashioned dumb-bells. The cytoplasm of the macrogametocytes of this parasite are not nearly so dark staining nor so alveolar as are those of the parasite of the pigeon. The pigment occurs in small parasites; in large ones it is plentiful and exists in coarse granules.

From these brief descriptions it appears that the "halteridia" we have described fall into three groups according to their characteristics. It is of course impossible without further study to affirm that these groups represent separate species.

REPTILES:

LIZARD (*Agama colonorum*): Trypanosomes.

Trypanosome (Fig. 43) occurs in preparations of blood made from a lizard at Vintang. It is a large, rounded parasite measuring from 23 by 27 microns to 35 by 12 microns. Its cytoplasm is coarsely alveolar and may contain several small vacuoles; it stains a deep blue. The kinetonucleus is small, compact, and densely staining. Adjoining the kinetonucleus, or partly surrounding it, there is often a small ill-stained oval area. Sometimes this area can be seen to be joined by a thread-like connection with the pale elongated area, which contains the chromatin of the nucleus. This elongated area is well-defined and it stains less deeply than does the surrounding cytoplasm; it may measure seventeen microns in length. The chromatophilic nuclear material consists of a loose-textured, faint-staining mass which lies at about the center of the elongated area in which it is placed. The undulating membrane is ample and there is a free flagellum.

A trypanosome of another type (Fig. 44) is present in the same slides. It is a smaller parasite, and it measures from 38 by 7 microns to 25 by 11 microns. Its shape is more or less oblong, but its posterior extremity may form a definite prolongation. The cytoplasm is almost homogeneous and it stains faintly; sometimes it stains pink. In the cytoplasm are a few vacuoles and very numerous red granules. The chromatin of the nucleus stains slightly and it lies in a clear elongated area, which may measure ten microns in length; the chromatin lies near the center of this area in a mass which may measure five microns in length. As in the larger trypanosomes, one end of the clear nuclear area may be closely connected with the densely staining kinetonucleus. There is an undulating membrane and a free flagellum.

A trypanosome identical with this in appearance, but sometimes measuring as much as 32 by 13 microns, was found in lizards of another genus (*Lygosoma* sp.).

The resemblance of both of these parasites to forms of *Trypanosoma loricatum* is evident.³ Trypanosomes, also

resembling forms of *Trypanosoma loricatum*, have been seen in lizards by Wenyon in the Sudan¹⁵ and by Martin¹⁶ in French Guinea.

Hemocytozoa.

Pigmented parasites harboring in the red blood cells of reptiles have been reported by several authors.^{12, 15, 17} One was seen in the blood of a Gambian lizard (*Agama colonomum*). Most of the forms seen were of comparatively large size (Figs. 45-48). None of the small forms described in the Sudan¹⁵ were seen; neither was the close connection between nucleus of the host cell and parasite, seen in our specimens, observed there. Larger forms (Figs. 49, 50) were infrequent; in these the chromatin was usually distributed peripherally, and it was divided into from eight to eleven masses. In some parasites (Fig. 50) one end is occupied by a mass of material taking the same stain as the chromatin. The texture of the cytoplasm of most parasites is loose and alveolar. All contain varying amounts of golden-brown pigment.

Some of the red blood cells of this lizard contain bacterium-like bodies (Figs. 51-53); they recall those which have been seen in the blood of frogs.

MONITOR (*Varanus niloticus*): Hemogregarine.

This monitor was shot at Lamin Koto, the blood films were made and the tissues preserved at once. No parasites were seen in the examination of the fresh blood. In the stained films there are a very few hemogregarines, all within red corpuscles. The largest are elongated, slightly curved bodies, 10.3 by 2.5 microns. The protoplasm stains very faintly and the cell outline is wrinkled. The nuclear material is densely stained and is placed in the middle of the parasite (Fig. 54). Most of the large forms are surrounded by a delicate pink stained capsule. Empty capsules in red cells occur in small numbers (Fig. 56) and probably are the result of damage done in making the preparations. No tapering or bent vermicular forms were seen. The host cell nucleus is often slightly displaced. The smallest parasites are oval in shape and measure about 6 by 3 microns

(Fig. 55). The protoplasm is finely alveolar, stains pale blue and often contains one or several chromatophilic granules. The nuclear material consists of a straight or curved rod-like body with rounded blunt ends, and lies along the periphery of the parasite. The host cell nucleus is usually displaced to one extremity of the corpuscle. Parasites, intermediate in size, were found. Most contain a large, oval nuclear mass of densely stained chromatin, centrally placed. In a few the nucleus appears to consist of a twisted skein-like mass of chromatin, and the parasites, in which this occurs, may be surrounded by a capsule.

Multiplication forms were found in all the organs; most abundantly in the lung, liver, and spleen. Forms enclosed in cells, probably endothelial cells, and corresponding to the micro- and macro- merozoites of other authors were found. The commonest number, determined by study of serial sections, of "micro-merozoites" appears to be sixty-four; of the "macro-merozoites" eight. Curved vermicular forms were found in pairs and fours enclosed in cyst membranes within endothelial cells. A more detailed description of the stages found in tissues will appear in another publication.

Hemogregarines in *Varanus niloticus* have been described by Laveran,¹⁹ Laveran and Pettit,²⁰ and Franca,²¹ in *Varanus griseus* by Nicolle and Comte,²² and in *Varanus arenarius* by Bouet.²³ In our case none of the folded vermicular forms in red cells described by Laveran¹⁹ and Laveran and Pettit²⁰ were found. The description and measurements given by Franca²¹ agree quite closely with ours, and it seems probable as he suggests that there are two species of hemogregarines to be found in *Varanus niloticus*. The comparison of the multiplication forms found in the tissues, to be described later, with those described by Laveran and Pettit²⁰ may furnish the required evidence.

DISCUSSION.

A list of the animals examined is given on pages 196 and 197; altogether fifty species and one hundred and seventy-five individuals, exclusive of man, were examined. The following

is a list of the parasites found in them together with the hosts in which they were found :

Trypanosome in a hornbill (<i>Lophoceros nasatus</i>).	
“ “ dove (<i>Streptopella vinacea</i>).	
“ “ bush fowl (<i>Francolinus</i> sp.).	
“ “ lizard (2 species)	{ <i>Agama colonorum</i> . <i>Lygosoma</i> sp.
Spirochetes in an antelope (<i>Hippotragus equinus</i>).	
Piroplasmata “ “ “ “	
Leucocytozoön in a bush fowl (<i>Francolinus</i> sp.).	
	{ <i>Vinago nudirostris</i> . <i>Streptopella vinacea</i> . <i>Turtur senegalensis</i> . <i>Numida meleagris</i> . <i>Lissotis melanogaster</i> . <i>Ptilopachus fuscus</i> . <i>Francolinus</i> sp. <i>Balearica pavonina</i> . <i>Neophron monachus</i> .
Halteridia in birds of 9 species	
A pigmented hemacytozoön in a lizard (<i>Agama colonorum</i>).	
Hemogregarines in a monitor (<i>Varanus niloticus</i>).	
Leucocytozooë in a vulture (<i>Neophron monachus</i>).	

In several instances parasites probably identical with these have been seen by other observers. It seems unnecessary to give every possible reference to their works, since these papers can be found so easily by an inspection of, for example, the Bulletin of the Pasteur Institute. Consequently, we have given references only when the point in question seems to demand it. Some of these parasites are probably described here for the first time. We still believe³ that protozoön parasites ought not to receive names until their specificity has been established; but for convenience of reference we suggest that the name of the host in which they were found be used as the specific name of the new parasites described in this paper.

The results of the few examinations on which this paper is based indicate once again that there is universal infection by protozoön parasites among animals inhabiting tropical Africa.

[We are indebted to Samuel Henshaw, Director of the Museum of Comparative Zoölogy, Harvard University, for the identification of most of the birds and small animals named in this paper.]

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EXPLANATION OF PLATES.

All the illustrations accompanying this paper have been drawn with the aid of the camera lucida. The objects were traced at the level of the desk top. The magnifications have been determined as accurately as possible, and are not uniform save for the series of parasites, with a few exceptions, from a given case. The dimensions given have been determined for each optical system used, both by tracing the stage micrometer (1 mm. in 100) upon paper with the camera lucida, and by means of a micrometer ocular. Zeiss apochromatic objectives 2 mm. and 1.5 mm. and compensating oculars 6, 8, and 12 were used.

In the reproduction the figures have been reduced .08 diameters from the original painting.

PLATE X.

Trypanosomes from Roan Antelope. (Page 200.)

FIG. 1. — Obj. 1.5 mm. and oc. 12. 3,660 diameters.

FIG. 2. — Obj. 2 mm. and oc. 12. 2,720 diameters.

Spirochetes from Roan Antelope. (Page 200.)

FIGS. 3, 4, and 5. — Obj. 1.5 mm. and oc. 12. 3,660 diameters.

Piroplasma. Roan Antelope. (Page 201.)

FIG. 6. — Parasite 1 micron in diameter. Round form.

FIG. 7. — " .6 by 1 micron. Dividing form?

FIG. 8. — " .8 micron long.

FIG. 9. — " 1.7 microns " "Bacillary form."

FIG. 10. — " 2.2 " " " " "

FIG. 11. — Two parasites in same corpuscle, 1.6 and 1.4 microns long.

FIG. 12. — Bigeminal form; individuals are 1.6 and 1.4 microns long.

FIG. 13. — Parasite 1.8 by 1.4 microns and chromatin body .6 micron in diameter.

Flagellated Organisms from Blood of Roan Antelope. (Page 201.)

The source of these may have been from the alimentary tract.

FIGS. 14, 15, and 16. — Obj. 1.5 mm. and oc. 12. 3,660 diameters.

FIGS. 17, 18, and 19. — Obj. 2 mm. and oc. 8. 1,860 diameters.

Leucocytozoores from Vulture. (Page 202.)

All drawn with obj. 2 mm. and oc. 8. 1,860 diameters.

FIGS. 20, 21, 22, and 23. — Parasites in mononuclear non-granular leucocytes.

FIG. 24. — Parasite in an eosinophilic leucocyte.

FIG. 25. — Parasite in a neutrophilic leucocyte.

Trypanosomes of Hornbill. (Page 203.)

Obj. 2 mm. and oc. 8. 1,860 diameters.

FIG. 26. — Large type.

FIG. 27. — Small type.

Trypanosome of Dove. (Page 204.)

FIG. 28. — Obj. 1.5 mm. and oc. 12. 3,660 diameters.

Trypanosomes of Francolin Bush Fowl. (Page 205.)

All drawn with obj. 2 mm. and oc. 8. 1,860 diameters.

FIG. 29. — Trypanosome with sharp posterior extremity.

FIGS. 30 and 31. — Trypanosomes with blunt posterior extremity.

Trichomonads from Francolin Bush Fowl. (Page 205.)

FIG. 32. — Obj. 2 mm. and oc. 8. 1,860 diameters.

PLATE XI.

Leucocytozoa of Francolin Bush Fowl. (Page 205.)

All drawn with obj. 2 mm. and oc. 8. 1,860 diameters.

FIGS. 33, 34, and 42. — Adult female parasites.

FIGS. 35 and 36. — Adult male parasites.

FIGS. 37, 38, 39, and 40. — Small or young forms. Fig. 39 probably represents a young male form.

FIG. 41. — Large rounded adult female parasite.

Trypanosomes of a Lizard. (Page 211.)

Both drawn with obj. 1.5 mm. and oc. 8. 2,440 diameters.

FIG. 43. — Rounded type.

FIG. 44. — Elongated type.

Hemocytosoma of a Lizard. (Page 212.)

All drawn with obj. 2 mm. and oc. 8. 1,860 diameters.

FIGS. 45, 46, 47, 48, 49, 50, and 51.

FIGS. 52 and 53. — Bacterium-like bodies in red cells of the same lizard.

Hemogregarine of a Monitor. (Page 212.)

All drawn with obj. 1.5 mm. and oc. 8. 2,440 diameters.

FIG. 54. — Large form, with dense skein-like arrangement of chromatin.

FIG. 55. — Small form.

FIG. 56. — Empty capsule remaining in red corpuscle.

PLATE XII.

Halteridia of Doves. (Page 209.)

All drawn with obj. 2 mm. and oc. 8. 1,860 diameters.

FIGS. 57 and 58. — Adult male parasites.

FIG. 59. — Adult female parasite.

Halteridia of a Stone Pheasant. (Page 210.)

All drawn with obj. 2 mm. and oc. 8. 1,860 diameters.

FIG. 60. — Smallest ring form.

FIGS. 61 and 62. — Young pigmented forms with divided chromatin.

FIG. 63. — Large pigmented form — female?

FIG. 64. — Large pigmented form — male?

Halteridia of a Vulture. (Page 210.)

Both drawn with obj. 2 mm. and oc. 8. 1,860 diameters.

FIGS. 65 and 66. — Adult forms.

Halteridia of a Pigeon. (Page 209.)

All drawn with obj. 2 mm. and oc. 8. 1,860 diameters.

FIG. 67. — Youngest form found.

FIG. 68. — Triple infection of a red corpuscle. Young forms.

FIG. 69. — Adult female parasite.

FIG. 70. — Adult male parasite.

Halteridia from Francolin Bush Fowls. (Page 210.)

All drawn with obj. 1.5 mm. and oc. 8. 2,440 diameters.

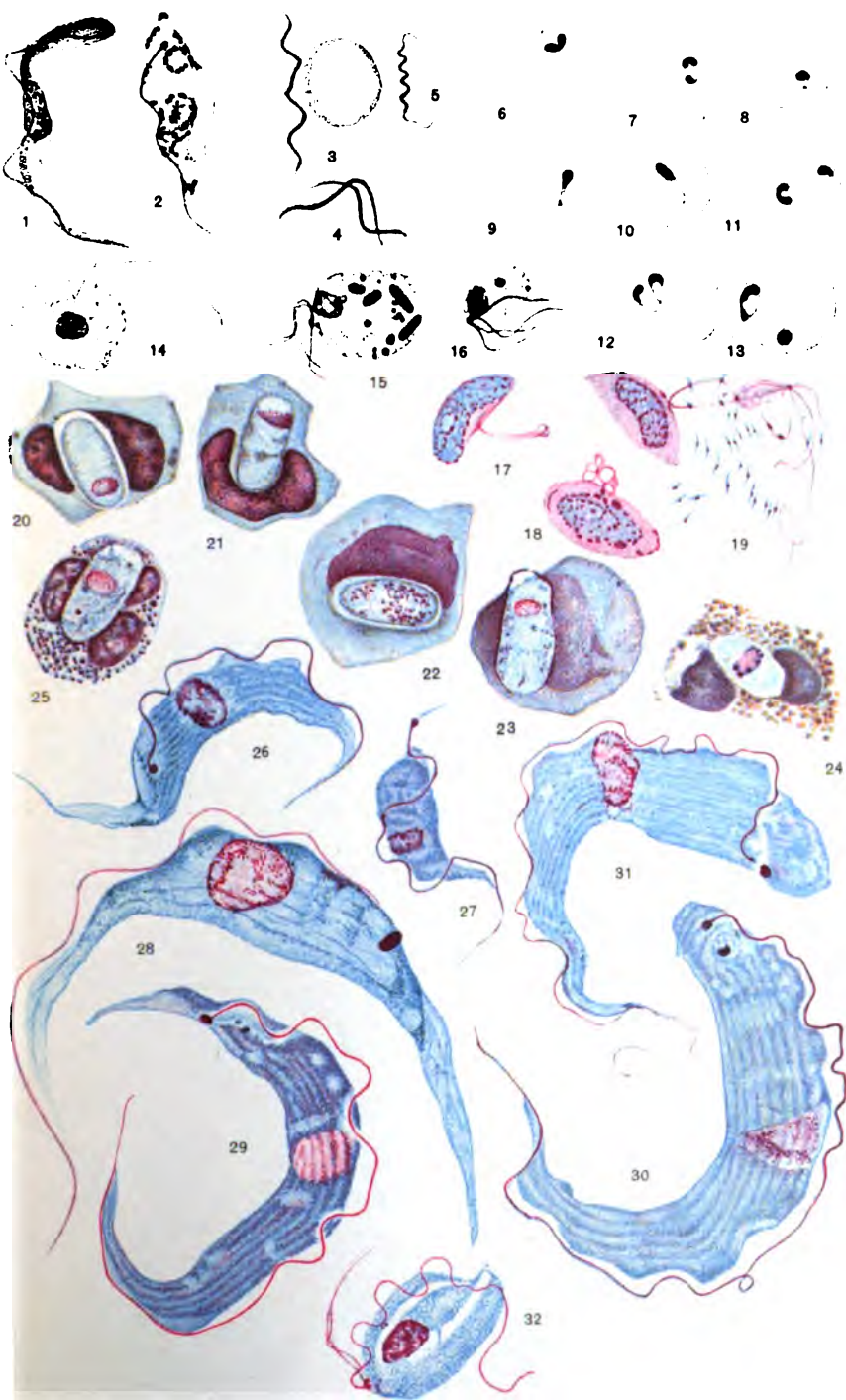
FIGS. 71 and 72. — Young form; sizes 1.4×1.8 microns and 1×2 microns respectively.

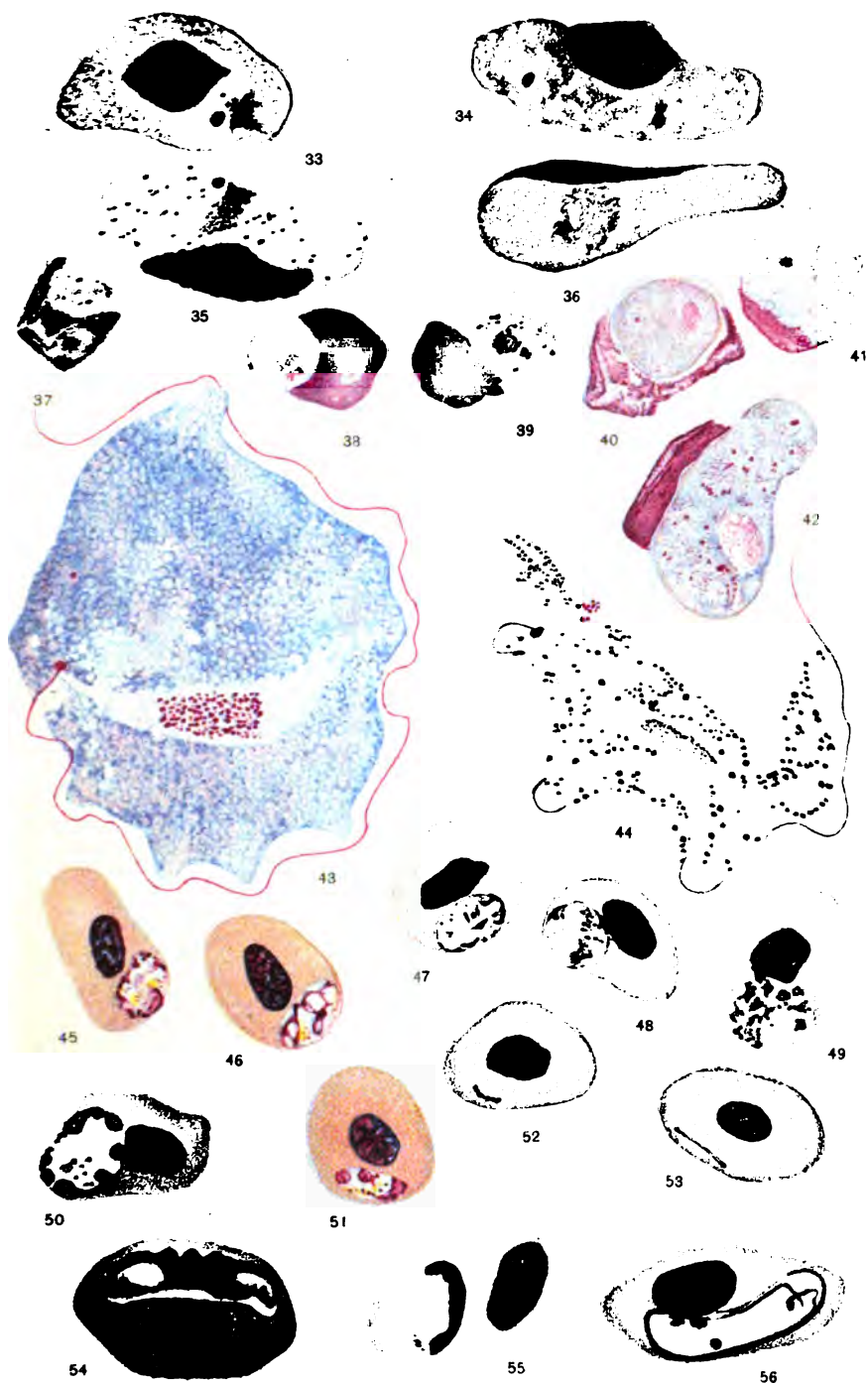
FIG. 73. — Lobed form. Size of each division .8 micron.

FIG. 74. — Young pigmented form. Size 1.8×2.2 microns.

FIG. 75. — Largest form seen. Size 2.2×4 microns.

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THE PATHOLOGICAL ANATOMY OF NATURAL AND EXPERIMENTAL MURRINA — A TRYPANOSOMAL DISEASE OF THE ISTHMUS OF PANAMA.*

S. T. DARLING, M.D.

(*Chief of Laboratory, Isthmian Canal Commission.*)

(*From the Board of Health Laboratory, Ancon, Canal Zone.*)

While the pathological anatomy of trypanosomiasis furnishes an interesting and extensive field for study, little has been done until recently, for the reason that demands for the investigation of methods of control and treatment have been more urgent. Baldwin,¹ Breinl,² Mott,³ Claude,^{4,5} and Renaud, Laveran,⁶ and Roudsky,⁷ with others, have contributed to the pathological anatomy of trypanosomiasis of various species; and Pettit⁸ recently has published some excellent observations on the transformation in the liver caused by eight species of pathogenic trypanosomes.

The object of this paper is to present some observations on the pathological changes elicited in a variety of animals naturally and experimentally infected with *Tr. hippicum*, Darl. This trypanosome is the cause of a fatal disorder among horses and mules in the Republic of Panama and the Isthmian Canal Zone. The disease is known locally by the names Murrina, Derrengadera and Moraña de Cadera.^{9, 10, 11, 12}

The observations are based on experiments with native horses, American mules and work horses, dog, raccoon, monkeys, — *Cebus hypoleucus*, *Nyctipithecus* sp., rabbits, guinea-pigs, rats, mice, and coati. Thus the pathological reactions in several classes of animals were determined.

The pathological anatomy of Murrina is of interest, not only from a scientific or systematic viewpoint, in which the various pathological reactions elicited by *Tr. hippicum* in a variety of animals are noted, but also from the point of view of the teacher, for it affords an excellent means for exhibiting the following characteristics of trypanosomiasis in general: Emaciation, edema and effusions; ecchymoses,

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petechiæ, and hemorrhages of the epi- and endo- cardium, peritoneum, pleura, and kidney; iridocyclitis, blepharitis, dermatitis, and conjunctivitis; anemia, lymphocytosis, auto-hemagglutination; hyperplasia of spleen, lymph nodes and bone marrow; phagocytosis of trypanosomes and red blood cells; various types of acute nephritis — interstitial, diffuse and glomerular — and hepatic necrosis.

Trypanosomiasis and spirochetosis of animals are deserving of far wider use in the experimental study of disease processes than is given them; and as a living, always demonstrable strain of trypanosomes may be kept in laboratory animals for months it lends itself well for purposes of demonstration.

In general, the pathological reactions in trypanosomiasis are characteristic, and the specific diseases will be found to have correspondingly specific lesions. To illustrate: iridocyclitis is one of the lesions caused by pathogenic trypanosomes, but certain species of trypanosomes provoke it only in certain genera of animals. Laveran mentions its occurrence in cats in Nagana. On the other hand, in Murrina it has been observed in *Nyctipithecus*, dogs, and the raccoon. Nephritis is encountered in all forms of trypanosomiasis and in most varieties of animals, but its type varies similarly. In Murrina, for example, the glomerulus is a very common point of attack in mules and horses, while in some of the smaller mammals the lesions are interstitial and tubular (monkeys and coati), while in still others only parenchymatous changes have been observed (guinea-pigs, rabbits, and rats). Widespread ecchymoses and petechiæ are usually seen in Nagana and Murrina, but never to the same extent in Mal de Caderas, Surra, or Dourine. In Surra the petechiæ appear to be confined to mucous surfaces. Blepharitis, conjunctivitis, and dermatitis of the ears are constant in Murrina rabbits, but were not observed in any other animal. On the other hand, Laveran reports that one of his Caderas guinea-pigs had a double blepharoconjunctivitis with opacity of each cornea. Conjunctivitis is common in Murrina horses and mules, but iridocyclitis

has never been observed in them. With regard to the nervous system, Sleeping Sickness, an infection by *Tr. gambiense* calls forth a perivascular, round-cell deposit in the brain. This cerebral lesion seems to be peculiar to man. It does not occur in Murrina. A lymphoid cell infiltration in necroses near the portal spaces of the liver studied recently by Roudsky and by Pettit, in the white mouse, has been seen in Murrina guinea-pigs, white mice, and one mule. A very striking change was seen in the spleen of the ant bear (coati). Normally, the spleen of this animal contains a few megakaryocytes, but no eosinophiles or other bone-marrow elements. After infection by trypanosomes, however, the spleen takes on, to an intense degree, some of the characters of red marrow, and it contains many megakaryocytes, nucleated red cells, and some eosinophiles.

Pathological anatomy. Mules and horses. — Pathogenic trypanosomal infections are associated with the production of and destruction of enormous numbers of these protozoa, and a study of the reactions caused by the trypanosomes suggests that a toxin is produced by the trypanosome, or host, which elicits the varied and widespread lesions in infected animals. Laveran¹⁸ and Pettit have recently shown that toxic symptoms may be produced in mice by the injection of saline filtrates of desiccated trypanosomes, *Tr. evansi*.

The particular group of lesions noted in horses and mules at autopsy depends on whether the animals were sacrificed or permitted to die of the disease, and also whether the animal died during the wet phase when there is edema and effusions, or during the dry phase when there is emaciation. Very few gross lesions are noted in animals sacrificed before the end of the disease other than emaciation, edema of the sheath, or lower belly wall, or, perhaps, some serous fluid in one of the cavities. Conjunctival or nasal ecchymoses are frequent, and there is some splenic enlargement. When a horse or mule is permitted to die of the infection, very characteristic lesions are found and they are fairly constant.

The spleen shows some enlargement and its capsule, particularly on the external surface, is dotted over with petechiæ one to two millimeters in diameter (Fig. 7). The more recent ones are brighter in color than the older, which are reddish-brown. The pulp is not perceptibly pigmented, and only slightly increased in amount. The reticulum is prominent. The rib and femur marrow are hyperplastic and the hyperplasia in the femur is not a diffuse one, but the marrow becomes cellular from the ends and periphery, gradually encroaching upon the yellow marrow in the interior. The kidneys are swollen and contain petechiæ in the cortex, about one millimeter in diameter distributed just beneath the capsule. The renal lymph nodes are usually enlarged and one or two of the sets are frequently the seat of extensive hemorrhage. The endocardium and epicardium usually contain large and small hemorrhages, often on papillary muscle or mitral leaflets; and in the epicardial fat along the coronary arteries, near the base of the heart (Fig. 11). The pleuræ and peritoneum have in some cases been the seat of numerous and extensive petechiæ. In the nasal and conjunctival mucosæ hemorrhages, usual petechial in size, are seen at some time during the disease. Sometimes there is an excessive amount of clear, serous, amber-colored fluid in the peritoneal, pleural, or pericardial cavities, but this is not constantly found at autopsy. Edema of the sheath, legs, hocks, or lower belly wall usually occurs during the disease, but is not constantly found at autopsy.

Horse No. 10, experimentally infected. — Duration of disease fifty-four days. Emaciation was extreme. The legs and belly wall were very edematous and the sheath was increased about four times its normal size. There was a considerable quantity of clear fluid in the peritoneal cavity. Beneath the pleuræ, near the apex of each lung, there were petechial hemorrhages, and many similar ones on the upper surface of the diaphragm. Along the coronary arteries of the heart and the auriculo-ventricular junction on each side, the petechiæ were so numerous that they made a striking picture. There were a few, also, on the endocardium. There was a perfect shower of petechiæ over the surface of the spleen. The kidneys were very pale and the petechiæ were extremely few in number and very small in size. The lymph nodes at the hilum of the kidney were soft and hemorrhagic. No ecchymoses in the conjunctiva were seen.

Naturally infected mule No. 305. — Sick about two weeks at Gatun. There was some emaciation, but no edema of the subcutaneous tissue. The latter was of a bright yellow color. In each pleural cavity there was a large excess of clear amber fluid. The lungs were congested but not consolidated. The heart muscle was pale and there was a large sub-endocardial hemorrhage on the mitral valve and one large epicardial hemorrhage. The cut surface of the liver was swollen and yellowish-brown in color. The spleen was slightly enlarged, but the pulp was not appreciably increased in amount. The capsule was dotted over with red and brown ecchymoses, about two millimeters in diameter. The kidneys were pale, swollen, and moist, with numerous sub-capsular petechiæ one millimeter in diameter. On the diaphragmatic portion of the peritoneum there were a few ecchymoses. The lymph nodes at the hilum of the kidneys were swollen, moist, and hemorrhagic. The intestines contained many nematodes and the stomach and intestines were full of chyme and fæces. The brain was negative.

Naturally infected mule No. 128. — Duration of disease not known. There was an excessive amount of amber-colored fluid in each pleural cavity. The subcutaneous tissue of the breast was dotted with small petechiæ, one millimeter in diameter, while the tissue along the vertebræ was dark yellow in color and edematous. The heart muscle was pale and the epicardial fat contained a few small ecchymoses, two millimeters in diameter. There was one large endocardial hemorrhage on a papillary muscle of the left ventricle and one on a mitral leaflet. The liver contained several cavernous hemangiomas. The spleen was slightly enlarged and there was a moderate number of red and brown capsular petechiæ, about two millimeters in diameter. The trabeculæ were distinct and the pulp did not bulge on section. There was a moderate number of petechiæ in the parenchyma of the kidneys just beneath the capsule, one millimeter in diameter. The deeper portions of the cortex were of a coarse yellow color, indicative of necrosis. One lymph node at the hilum of a kidney was slightly swollen and red. The bladder contained normal urine.

Naturally infected mule No. 359. — The body was emaciated, the right hind leg was slightly edematous, and there were a few ulcers on the hock. There was a slight excess of clear amber fluid in the peritoneal cavity. The underlying lung was edematous and congested, and contained much frothy serum in the bronchioles which had normal mucosæ. The other lung was normal. There was one area of hemorrhage in a subpleural emphysematous bulla, one and one-half centimeters in diameter. The heart contained several small ecchymoses in the epicardium over the ventricles and at the apex. The endocardium of each ventricle contained many large hemorrhages. The liver was negative. The spleen was slightly enlarged and there was a moderate number of red and brown capsular ecchymoses, one to two millimeters in diameter. The trabeculæ were prominent, but the pulp was not increased in amount or diffuent. The

red bone marrow was dark red and moist, but not appreciably hyperplastic. In the kidneys there were numerous subcapsular ecchymoses, one millimeter in diameter. A few were deeper in the cortex. The parenchyma was moist and pale, with no evidences of necrosis or exudation. The ureters and the pelvis of the kidneys were thickened and the mucosæ covered with tenacious mucus. The renal lymph nodes were congested and edematous. The bladder was contracted and its wall thickened (the animal had suffered from incontinence of urine). The stomach and intestines contained many nematodes.

Naturally infected mule No. 263. — Emaciation was very marked and there were conjunctival ecchymoses in both eyes. The right pleural cavity contained a fibrinous clot. There were epicardial and endocardial ecchymoses. The spleen presented many capsular ecchymoses. The kidneys were negative.

Naturally infected mule No. 382. — The animal was very sick and was sacrificed. There was emaciation but no edema. The heart contained no endocardial nor epicardial hemorrhages. There were, however, many capsular petechiæ on the surface of the spleen. The splenic spaces were not engorged and the organ was not appreciably enlarged. The kidneys contained many petechiæ beneath the capsule and the renal lymph nodes were swollen and red.

Naturally infected mule No. 257. — There was emaciation but no edema. The heart contained epicardial and endocardial hemorrhages of large size. There were also some small ecchymoses in the epicardium of the first portion of the aorta. The liver was negative. The external surface of the spleen had many brown and red ecchymoses, but the internal surface none. The spleen was slightly enlarged, but the pulp was not appreciably increased in amount. The kidneys contained no visible petechiæ, but the parenchyma was pale, swollen, and moist. The lymph nodes draining the kidneys were swollen and of dark color. One was greatly enlarged and friable, and contained a large area of hemorrhage. The peritoneum was negative.

Naturally infected mule No. 185. — The animal was extremely emaciated. There were a few ecchymoses on the palpebral conjunctivæ. There was no edema of the legs, sheath or belly wall. The heart had many epicardial ecchymoses along the coronary arteries. There were a few ecchymoses on the diaphragm, and many petechiæ in the capsule of the spleen. The kidneys contained numerous petechiæ and there were hemorrhages in the renal lymph nodes.

Naturally infected mule No. 204. — This animal had been in a sling for two weeks and as he was unable to stand he was sacrificed. Emaciation was moderate and there was no edema. Subconjunctival ecchymoses

were present. Several excoriations were noted corresponding to points of pressure of the halter or sling. The heart contained no endocardial nor epicardial hemorrhages (the animal was shot). Spleen contained a few capsular ecchymoses and the kidneys several petechiæ on the cut surface.

Naturally infected native pony No. 397. — This was the first native horse I had had an opportunity of examining post-mortem, and from this case I became convinced that the disease was an endemic one and had not been introduced from the United States, or elsewhere.

The animal was emaciated but there was no edema. It is said that his haunches were markedly weakened and that he had been "down" for several days. The lesions noted corresponded closely with those seen in the American mules and work horses: Sub-endocardial ecchymoses, epicardial ecchymoses, sub-capsular renal petechiæ, diaphragmatic ecchymoses (peritoneal), and splenic capsular ecchymoses.

Pathological anatomy — other experimentally infected mammals. — A calf, hog, cat, and agouti proved refractory, or recovered from the infection, so that no opportunities were presented for noting lesions in these animals, beyond the fact that a cat had dermatitis of both ears.

Monkeys: *Cebus hypoleucus* and *Nyctipithecus felinus* (?). — In *Cebus* the spleen was greatly enlarged — the typical spleen of anemia. The splenic pulp was greatly increased in amount and the capsule was very tense. The heart muscle was edematous and there were a few epicardial ecchymoses. Anemia of viscera was extreme.

In *Nyctipithecus* the spleen was also greatly enlarged. Each eye in its anterior chamber contained a large, gray, flattened flake of fibrin. The liver showed advanced fatty metamorphosis.

Dogs and raccoons showed nearly identical lesions. Each eye contained a glistening, opaque, grayish-yellow flake of fibrinous exudate in the anterior chamber, while the scleræ were perfectly clear. The spleen was much enlarged, and the Malpighian bodies large and prominent. The pulp was gray in color and the capsule contained no petechiæ. The kidneys were enlarged and the parenchyma swollen, very moist and yellowish-gray in color. Beneath the capsule the surface was mottled with ecchymoses and light gray necroses, or areas of exudation. The bladder contained many submucous hemorrhages. In the lungs there were several hemorrhages two to three millimeters in diameter. In the raccoon there was an ulcer behind each last upper molar. It was covered

with a diphtheritic exudate and probably corresponds with Crombie's molar ulcer.

An opossum showed no gross lesions.

In the coati, *Nasua narica*, the spleen was enlarged and uniformly dark colored and on the posterior part of the epicardium was a thin fibrinous exudation.

Rabbits. — In these animals there was during the disease a scaly dermatitis of the ears, edema of the muzzle and eyelids, with blepharitis and conjunctivitis, followed by alopecia. The spleen of one rabbit was very much enlarged at the time of death, while that of the other was normal in size. A few petechiæ were seen in the capsule of the spleen, there was one hemorrhagic lymph node, and some emaciation.

Guinea-pigs. — Edema of the external genitalia and ears with ulceration of the skin of the scrotum was seen. The spleen was usually enlarged.

Rats and mice. — There was great enlargement of the spleen in all of these animals, the pulp being greatly distended and contained large, yellow patches of necrosis.

Histopathology of equines:

Mule No. 531. — Liver: This animal was naturally infected and was scarified by bleeding. The sinusoids contain considerable bronze pigment, which has been phagocytized by endothelium and circulating mononuclear cells. There are here also many polymorphonuclear and mononuclear leucocytes, and some very small necroses, limited to one or two hepatic cells, in which the latter have been replaced by polymorphonuclear and mononuclear leucocytes. In one portal space there is a large collection of cells which occupies an area about one-fourth of a lobule. The cells have replaced the peripheries of two adjoining lobules, and have come from the blood stream, for the sinusoids, hepatic and portal veins contain many others like them. Most of these cells are mononuclear leucocytes of the intermediate type, and about fifteen to twenty per cent are polymorphonuclear. The mononuclear cells have deeply staining plastic nuclei with irregular outlines, and several show mitoses. They are evidently proliferating in their new location. Extending out into Glisson's capsule from this patch are smaller lines of necrosis and infiltration by lymphocytes. The hepatic veins are crowded with leucocytes and large round, oval and fusiform collections of bronze, refractile pigment, enclosed in circulating phagocytes and endothelial cells. There are slightly more polymorphonuclear than mononuclear cells, and the latter are of several types, some having large, spherical, vesicular nuclei and others elongated plastic nuclei. No hyaline central zone necroses are seen, but there

are a number of linear and small peripheral zone necroses, accentuated near some of the portal spaces and also dotted throughout the lobule.

Lymph node: There is diffuse hyperplasia of the dense lymphoid tissue, the intermediate and large mononuclear type cells predominating. Many of the cords and nodes contain large mononuclear cells with ragged cytoplasm and large vesicular nuclei about ten microns in diameter. There is a polymorphonuclear increase in the arterioles, but no eosinophilia. A few collections of bronze blood pigment are seen in all parts of the node, even in the germinal centers of the follicles.

Spleen: The Malpighian bodies are of large size and the pulp contains a greatly increased number of large mononuclear cells. Each Malpighian body has a large, dense, deeply staining core, consisting of large-sized mononuclear cells, the latter having pale nuclei. Toward the periphery of the body the cells are less closely packed and have relatively more cytoplasm. This border is very deep and shades off into the pulp, giving the Malpighian bodies an unusually large appearance. The peripheries of the Malpighian bodies are made up of completely developed, large mononuclear leucocytes, exactly like the large circulating phagocytes seen in the liver. The extreme periphery contains a number of polymorphonuclear and mononuclear leucocytes, the latter with irregularly shaped, elongated and plastic nuclei resembling the polyblasts of Maximow, such as are seen in the large portal areas of necrosis of the liver. The splenic spaces are not distended by red blood corpuscles, but they do contain large numbers of mononuclear and polymorphonuclear cells, containing much bronze refractile blood pigment. There is no eosinophilia and no cells of the megakaryocyte type.

Kidney: Here and there beneath the capsule are small spots of hemorrhage in which the tubules are filled with red blood cells. The interstitial tissue is not involved. Near these areas of hemorrhage and scattered throughout the organ, particularly among the straight tubules, are cellular casts composed chiefly of polymorphonuclear leucocytes with some red blood cells. In the neighborhood of the large vessels and close beside some of the glomeruli are collections of round cells, similar to those seen in the liver. Some have large translucent nuclei, while others have elongated, plastic, deeply staining nuclei, and resemble cells seen in the spleen, liver sinusoids and hepatic necroses. The arterioles contain an increased number of polymorphonuclear and mononuclear leucocytes, and some show mitotic figures. The tubular epithelium appears normal on the whole, but a few tubules have undergone necrosis. The glomeruli are distended by polymorphonuclear and mononuclear leucocytes, some of which have undergone pycnosis and karyorrhexis.

Mule No. 359. — Liver: The hepatic cells are cloudy and the nuclei obscure. The sinusoids are dotted along their course by large collections of brownish-red, refractile blood pigment (Fig. 9). The leucocytes are increased in number, and here and there a hepatic cell has disappeared and its place is taken by two or three polymorphonuclear leucocytes.

Kidney: There are some intertubular hemorrhages and the lumina of a number of the tubules contain masses of red blood cells. Some of these are recent, while others show disintegration. There is some polymorphonuclear leucocytic exudation into the tubules in association with the hemorrhages. The tubular epithelium is generally cloudy, but the glomeruli everywhere appear normal.

Spleen: The splenic spaces are not distended but are moderately filled with cells of the large mononuclear type, many of which have phagocytosed red blood cells (Fig. 8), and many others contain brownish-yellow, refractile blood pigment. The amount of phagocytosis of blood cells and pigment is enormous.

Lymph node: Scattered here and there are large areas of hemorrhage into the loose lymphoid tissue, and in these places there is considerable phagocytosis of red cells by large mononuclear cells.

Mule No. 128. — Liver: There is the usual amount of phagocytosed, reddish-brown, refractile blood pigment in the sinusoids, which are generally wider and contain more leucocytes than usual.

Kidney: There is some edema and the intertubular connective tissue is slightly increased in amount, as well as in Bowman's capsule. There are hemorrhages and leucocytic tubular casts as in No. 359. The glomeruli everywhere appear normal.

Lymph node: This contains a number of hemorrhages and much phagocytosis of blood pigment.

Omentum: This contains a recent hemorrhage with extensive leucocytic infiltration into one portion.

Mule No. 546. — Liver: The endothelium of the sinusoids does not contain as much phagocytosed blood pigment as usual, and there is some deposit of fat in the liver cells.

Kidney: Moderate, acute glomerulitis with infiltration of glomerulus by polymorphonuclear and mononuclear leucocytes, and small points of necrosis. The tubular epithelium is cloudy, but there are no areas of leucocytic exudation here and no hemorrhages.

Renal lymph node: There is diffuse hemorrhage into the loose lymphoid tissue (the autopsy picture in this case was not at all typical, but the histopathology is that of Murrina).

Mule No. 382. — Kidney: There is diffuse cloudy swelling of the tubular epithelium with the usual focal hemorrhages, and there is a well-marked acute glomerulitis in which every glomerulus is the seat of a polymorphonuclear and mononuclear leucocytic infiltration. The glomeruli are distended, there is proliferation of the endothelial cells of the tuft and several spots of necrosis are seen (Fig. 10). The tubular portion of the kidney, excepting quite near a glomerulus, shows no evidences of leucocytic exudation.

Spleen: The Malpighian bodies are of good size and there is the usual amount of blood pigment and of phagocytosed red blood cells in the splenic spaces.

Lymph node: The node is normal.

Mule No. 305. — Kidney: The usual picture of focal hemorrhages, acute glomerulitis, cloudy swelling and a few leucocytic tubular casts.

Liver: The hepatic cells are full of bile pigment, the sinusoids present the usual leucocytosis, and there is an unusual amount of very dark blood pigment, phagocytosed, in clumps. **Lymph node:** Extensive edema and hemorrhage.

Brain (Hippocampus major and cerebral cortex): There are no perivascular cell collections as in human trypanosomiasis. The section is quite normal.

Omentum: A number of small linear areas of hemorrhage. **Adrenal:** Negative. **Spleen:** The spaces are not distended, but there is a very large amount of blood pigment in large brown and black clumps. There are also many evidences of the phagocytosis of red blood cells and much glistening brown pigment.

Endocardium: Two large hemorrhages in which the blood has become laked and in which there is no exudation nor organization.

Mule No. 363. — Kidney: There are some interstitial hemorrhages and a few of the glomeruli contain leucocytes and mononuclear phagocytes with brown glistening pigment. There is cloudy swelling of some tubules, and epithelial necrosis in others. A few hyaline casts and a few leucocytic plugs are seen in some of the tubules.

Spleen: The Malpighian bodies are of good size and the splenic spaces are filled with phagocytosed obsolescent red blood cells and blood pigment. Nothing suggestive of a myeloid change is seen.

Liver: Most of the lobules exhibit some atrophy of the columns of liver cells in the central zone and some large central areas of hyaline necrosis. There are also small points of necrosis in which polymorphonuclear leucocytes are phagocytosing the defunct hepatic cells. The sinusoids everywhere contain an increased number of polymorphonuclear leucocytes, phagocytosed blood pigment and phagocytosed red blood cells in all stages of decline. In the areas of central zone necrosis the hepatic cells are cloudy or hyaline, and the nuclei swollen or obscure. There is some phagocytosis in these areas by polymorphonuclear leucocytes, but the necrosis is primary and the phagocytosis secondary. The red blood cells stain well and there are no hyaline or fibrinous thrombi here.

Lungs: Negative. **Brain, cerebral cortex:** Negative. **Heart:** There are some small patches of hemorrhage in the endocardium. Some of them are old and the blood is laked. But there is no exudation or organization. **Lymph node:** This node is greatly distended by serum which has coagulated everywhere, leaving a fibrinous reticulum. The loose lymphoid tissue is greatly distended by the coagulum in the meshes of

which are lymphocytes of various types, the most prominent being very large phagocytes with single vesicular nuclei. These cells have phagocytosed polymorphonuclear leucocytes chiefly.

Mule 132. — Brain, myocardium and adrenals: Negative.

Liver: The sinusoids contain many polymorphonuclear leucocytes and blood pigment. There are some very small areas of necrosis involving a few hepatic cells, in which there is a polymorphonuclear leucocytic infiltration.

Spleen: The usual picture of phagocytosed blood pigment.

Lymph node: Several areas of hemorrhage and some hyperplasia of lymphoid cells. There is a great deal of phagocytosis of red blood cells by large mononuclear cells.

Naturally infected native pony No. 397. — Spleen: The splenic spaces are not distended and the Malpighian bodies are not differentiated from the rest of the pulp. The cellular elements of the latter are increased in amount, due chiefly to a proliferation of large mononuclear cells. Very rarely a megakaryocyte is seen, but no eosinophiles were detected. There are very few collections of blood pigment.

Liver: There are a few collections of phagocytosed blood pigment and a slight but definite increase in the number of polymorphonuclear leucocytes in the sinusoids, as well as collections of bodies resembling trypanosome residue phagocytosed by endothelial cells.

Kidney: The endothelial cells of some of the glomeruli are swollen and the tufts contain an increased number of polymorphonuclear leucocytes. The tubular epithelium is slightly swollen, many of the nuclei are pycnotic, and there is a slight amount of epithelial necrosis.

Experimentally infected native pony treated with arsenious oxide. — Adrenal: Tremendous distention of blood sinuses in the cortex (the gland was a homogeneous deep reddish brown color) and there is much hyperplasia of the medulla.

Femur marrow: As noted in the gross specimen, the central portion of the marrow is pure yellow marrow substance, but toward the periphery one encounters more and more red myeloid elements. One megakaryocyte was seen in which the nucleus suggested karyokinesis. There are many megakaryocytes, myeloblasts and nucleated red cells. There are also collections of phagocytosed, moribund red blood cells.

Histopathology of other mammals:

Cebus hypoleucus. — Spleen: The splenic spaces are distended by red blood cells and by large round and oval phagocytes, eighteen to thirty microns in diameter, which have engulfed red blood cells and normoblasts. The spaces also contain a large number of mononuclear cells of several types and sizes. In some places there are small collections

of neutrophilic polymorphonuclear leucocytes, but there is no destruction of tissue. Blood pigment was not detected.

Kidney: There are a few interstitial hemorrhages, some of which are associated with the presence of hyaline tubular casts. The tubular epithelium is generally normal, but in a few places the cells are swollen, granular, eosinophilic, and have small pycnotic nuclei, rarely a glomerulus contains a few cells of the polymorphonuclear or the mononuclear type with excentric nuclei. There are also small interstitial collections of cells, chiefly of the mononuclear excentric nuclei type, and a few polymorphonuclear leucocytes, all of which have wandered out of the capillaries, oftenest near a glomerulus. They have destroyed the epithelium of the tubules by phagocytosis, and in a few instances they can be seen entering a tubule from the capillaries. The veins contain fibrinous thrombi and an excessive number of leucocytes, the mononuclear type predominating.

Nyctipithecus. — Liver: Many of the portal spaces are surrounded in an irregular and incomplete manner by collections of lymphoid cells which have replaced the hepatic cells. These lymphoid cells are mononuclear in type, with plastic nuclei and some have two or three nuclei. The nucleus is always vesicular and plastic and the cells are exactly like the mononuclear cells in the sinusoids. All of the central zones of the lobules are paler than the rest of the lobule and there is much cloudy swelling and fatty change in the hepatic cells in the central zone. The nuclei are swollen and pale and although the sinusoids contain a great many polymorphonuclear and mononuclear cells, the latter have not actually invaded, nor occupied, the hepatic cells of the central zone. The portal veins and sinusoids contain a great many large mononuclear and polymorphonuclear cells. The endothelial cells of the sinusoids are frequently enormously swollen. Their nuclei are very pale and some of them contain pale granules of doubtful nature. The hepatic cells everywhere are cloudy. There are two types of necrosis here, the central non-inflammatory and the smaller focal necroses associated with mononuclear phagocytes. Some of these latter are very large, but none are certainly megakaryocytes.

Kidney: The tubular epithelium has undergone a granular necrosis and the nuclei are either obscure or pycnotic. There is an extensive infiltration of the intertubular tissue by cells of an intermediate lymphocyte type. The nucleus is four to eight microns in diameter, the cells are round, oval or have ragged margins. They are ameboid in appearance and cannot be differentiated from similar cells in the blood vessels of the kidney. They are evidently brought by the circulating blood where they wander out of the intertubular capillaries and are seen to invade and remove large portions of the tubular epithelium. This interstitial mononuclear cell exudation appears to be identical with that described by Councilman in scarlet fever.

Spleen: There is great distention of the splenic spaces by several kinds of cells. The Malpighian bodies are not well differentiated from the

rest of the pulp, but there are small collections of lymphoid cells scattered everywhere. Only one typical Malpighian body is seen. This is surrounded by a dense collection of mononuclear cells with plastic nuclei and ameboid cytoplasm, exactly like the cells seen in the kidneys and liver. The splenic spaces present an extraordinary appearance, for they are filled with very large mononuclear phagocytes. They have engulfed red cells, polymorphonuclear leucocytes, and occasionally other mononuclear cells. Their cytoplasm is always turbid and in some instances they appear to have occluded the splenic spaces. In one area in which polymorphonuclear leucocytes predominate a few megakaryocytes are seen, but no eosinophiles.

Eye: The lesions are identical with those of the raccoon.

Raccoon. — Liver: There are no collections of cells around the portal spaces, nor in any part of the lobule. The hepatic cells in places are slightly hyaline, while others show very marked hyaline degeneration with fatty change. The nuclei of the hepatic cells are generally swollen and pale — rarely one is pycnotic or shows mitotic figures. The sinusoids contain many collections of black, blood pigment granules enclosed in endothelial cells. The leucocytosis is very slight. In the vessels a number of basophilic red blood cells and a few normoblasts are seen.

Spleen: The Malpighian bodies are not very conspicuous for the reason that their constituent cells have an unusually large amount of cytoplasm, differing in this respect from the hyperplastic bodies of the monkey and guinea-pig. The pulp is not engorged with red blood cells, but shows a considerable increase in the number of large lymphocytes. Polymorphonuclear leucocytes are increased in number, but there are no eosinophiles nor megakaryocytes. A few granules of blood pigment are seen and a few bodies, some of which have been phagocyted, are suggestive of trypanosome residues.

Kidneys: The glomeruli contain small clumps of polymorphonuclear leucocytes encased in hyaline material. The tubular epithelium presents all changes from cloudy swelling, with various transitions, through hyaline to fatty change. The nuclei of the epithelial cells are swollen, obscure, pycnotic or absent. Some of the tubules are widely distended and their epithelium flattened and atrophied, numerous hyaline granular and cellular casts are seen. The latter are made up of neutrophilic and eosinophilic leucocytes, large mononuclear leucocytes and hyaline epithelial cells. The most striking change is the presence of interstitial collections of cells with plastic nuclei of the large mononuclear, small lymphoid and epithelioid type, as well as neutrophilic and eosinophilic leucocytes of the polymorphonuclear type. These interstitial collections of cells correspond with cells seen in the capillaries and blood vessels nearby, and may be seen escaping into the interstitial tissue and occasionally into the tubules, the epithelium of which they replace. Many of these wandering cells have very plastic outstretched nuclei of bizarre shape. Some of them have a nuclear pseudopodium and they are actively ameboid. The glomerular changes are in striking contrast with the intertubular on account of the absence of eosinophiles and mononuclear cells in the former.

Eye: Between the sclera and the anterior surface of the lens is a shrunken irregularly flattened mass of fibrin attached in one or two places to the posterior surface of the sclera. This mass of fibrin has entangled a good many polymorphonuclear leucocytes and a number of squamous epithelial cells. The periphery of the flake of fibrin is richly dotted over with polymorphonuclear leucocytes. The desquamated epithelial cells are sometimes single, but frequently have slipped off in flakes containing eight or ten cells. The sclera is free from leucocytes except for a very thin layer over its posterior surface. The pigmented iris contains a great many medium sized lymphoid cells of the plasma cell type, together with a smaller number of polymorphonuclear leucocytes. The arterioles are filled with polymorphonuclear and mononuclear leucocytes and a very few red blood cells. The endothelial cells of the arterioles are very much swollen and leucocytes can be seen passing through the walls. The surface of the ciliary body shows an exudation of various leucocytes, chiefly polymorphonuclear. Here and there are collections of two or three to a dozen deeply staining bodies, free or intracellular, in what appear to be the fixed cells of the body. They are very suggestive of trypanosome residues. The arterioles of the fringe of the ciliary body are free from leucocytes. In the ciliary body near the attachment of the iris a few capillaries contain an excessive number of leucocytes. There is also an interstitial increase in cells of the plasma cell type. The distinctive lesion is in the iris and anterior chamber.

Coati. — Liver: There is no cellular proliferation or exudation around the portal spaces nor in any part of the lobule, but there has been a great loss of substance in the intermediate zone, for many of the hepatic cells have disappeared leaving a few islands of swollen, hyaline, hepatic cells. In other zones, however, there has been no necrosis and the hepatic cells contain many dark pigment granules, probably related to bile. The sinusoids are engorged with blood and they contain a slight increase in the number of polymorphonuclear leucocytes.

Spleen: The Malpighian bodies are not appreciably enlarged, but the splenic spaces are distended by red blood cells and by an increase in the number of lymphoid cells. The Malpighian bodies are made up of large, round, and oval lymphocytes, with a few epithelioid cells and eosinophiles of the polymorphonuclear and myelocyte type. The pulp presents an unusual appearance on account of its resemblance to red marrow, for it contains many eosinophilic myelocytes and megakaryocytes (Fig. 3). Signs of great activity are seen. The walls of the sinuses are made up of cuboidal cells, having an eosin staining fibrillated cytoplasm, the nuclei of which frequently show mitoses. Lymphoid cells can be seen making their way through the walls of the sinuses. Megakaryocytes are seen in the wall or lumen of a sinus. The sinuses contain at times nothing but red blood cells; at others large lymphoid cells and eosinophilic polymorphonuclear and myelocytic leucocytes, while others contain all of these and in addition what appear to be ghosts of basophilic degenerated red blood cells and trypanosome residues.

When the normal spleen of this animal is compared with one in trypanosomiasis it is noticed that the volume of the former is much smaller than that of the latter, and that the Malpighian bodies are correspondingly smaller. Similarly, there is less lymphoid hyperplasia in the normal spleen (Figs. 4 and 5). The general architecture is about the same, though the acinus-like splenic spaces which showed signs of such activity in the trypanosome infected animal are here made up of spindle or endothelial-like cells, with some transitions into the cuboidal type. In the normal spleen there is no eosinophilia, but there are numerous nucleated red cells and rarely a megakaryocyte.

Guinea-pig. — Liver: Each portal space contains a collection of cells with large round or oval vesicular nuclei and little cytoplasm, a few epithelioid cells and polymorphonuclear leucocytes, but no eosinophiles nor megakaryocytes (Fig. 1). These lymphoid cells surround the vessels and bile ducts and are several layers thick. Besides these portal collections, others are seen in every zone as well as beside the hepatic vein. The hepatic cells show extensive and diffuse hyaline change and each cell contains a large amount of fat. The nuclei of the hepatic cells are swollen and vesicular. In some spots the hepatic cells have entirely melted away, and here there are areas of hemorrhage and some leucocytic infiltration. The sinusoids contain collections of agglutinated trypanosomes or their residues all along their courses, and in addition an increased number of polymorphonuclear leucocytes and mononuclear phagocytes. These latter have engulfed trypanosomes, and their cytoplasm is hyaline.

Spleen: There is hyperplasia of the Malpighian bodies. The splenic spaces are filled with red blood cells, clusters of trypanosomes, a great variety of cells of the lymphoid type and polymorphonuclear leucocytes. A few of the mononuclear cells are small lymphocytes in type, but by far the greater number are large cells with round or oval vesicular nuclei, some of which show mitotic figures. These latter are most numerous in the Malpighian bodies, which are almost entirely composed of them. In addition, there are a few megakaryocytes, but no eosinophiles. In addition to other elements the splenic spaces contain a number of large mononuclear cells which have engulfed trypanosomes, nucleated remnants and red blood cells.

Ear: The ear had often been pinched and cut while making blood smears and this trauma may have had some influence in determining the lesions noted. There is a very striking large perivascular collection of lymphoid cells and some with plastic nuclei. They are to be seen making their way through the vessel wall. These collections of cells extend along each side of the ear cartilage and invade the striated muscle in some places. The skin has remained normal.

Testicle: Essentially the same change noted in the ear, but there has been more exudation and some atrophy of the epithelium and ulceration. Some of the striated muscle fibers have atrophied and are infiltrated by leucocytes. Deep sections show peri- and epi-neural round cell exudation.

The corpus cavernosum contains a greatly increased number of polymorphonuclear leucocytes. There are extremely few trypanosomes uniformly distributed in the blood of the corpus cavernosum compared with the number in the peripheral blood at the time of death, but this is accounted for by the very large amount of phagocytosis of trypanosomes and red blood cells and of agglutination of trypanosomes caught in fibrinous tangles in the blood sinuses.

Rat. — Liver: The sinusoids contain an excessive number of polymorphonuclear leucocytes. There are many trypanosomes but no blood pigment. A number of small areas of necrosis involving about a dozen hepatic cells are seen. Here the hepatic cells are more or less completely replaced by mononuclear and polymorphonuclear leucocytes. These necroses are in all zones of the lobules. In addition, there are many lobules in which the central part of the intermediate zone has undergone coagulative or hyaline necrosis with fatty change (Fig. 6). The nuclei of the hepatic cell are pycnotic, usually much swollen and fragmented. Elsewhere the nuclei of the hepatic cells are swollen. The vessels, particularly the smaller ones, contain many tangles of trypanosomes. There are no collections of lymphoid cells.

Spleen: The Malpighian bodies are not very well defined for there is considerable diffused lymphoid hyperplasia. The splenic sinuses are filled with red blood cells, agglutinated trypanosome residues and many large cells of the endothelial type, which have undergone hyaline change. Their nuclei, usually round or oval, frequently show pycnosis, karyorrhexis and giantism. These cells often contain fine granules of bronze blood pigment, or rarely coarse granules, such as is seen in the horse. A few megakaryocytes are seen. There is much necrosis of individual cells throughout the spleen.

Kidney: Cloudy swelling and a small amount of epithelial necrosis of the tubular epithelium. In neighboring epithelial cells there is giantism of the nucleus and pycnosis.

Mouse. — Liver: The columns of hepatic cells are atrophied and the sinusoids are filled with polymorphonuclear and mononuclear leucocytes, red blood cells, and trypanosomes. The nuclei of the hepatic cells are greatly enlarged and pycnotic. Some of the hepatic and portal veins are surrounded by collections of polymorphonuclear and mononuclear leucocytes thirty to forty microns wide. These have replaced the hepatic cells and may be seen passing through the vessel walls. Scattered over the lobules, without regard to zone, are many smaller areas of necrosis of hepatic cells and their replacement by leucocytes. Eosinophiles and megakaryocytes are absent. The vessels are all filled with agglutinated trypanosomes.

Spleen: The increased volume of the spleen is due to the engorgement of splenic spaces by red blood cells. There are numerous large collections of polymorphonuclear leucocytes in the pulp and some show considerable nuclear fragmentation. There is much hyaline necrosis in

all portions of the spleen except the Malpighian bodies. Eosinophiles are absent but the megakaryocytes are definitely increased in number, there being a small number normally in the spleen of this animal.

Kidney: No change beyond an infection by *Klossiellia muris*.

Hemosiderin reaction.—Fresh tissue, smears, and sections of tissue, when treated for iron containing pigment, show the presence of hemosiderin in considerable amounts in certain viscera, particularly the spleen. In the mules and horses and some of the infected small mammals, the spleen at the time of death contained large amounts of hemosiderin. The liver, heart, kidneys and bone marrow contained much less and this was seen chiefly in the connective tissue lymph spaces around the vascular trunks. The portal spaces in the liver took the iron stain deeply, while the parenchyma, to gross appearance, contained no hemosiderin. Microscopically, the liver sinusoids contain large greenish-blue clumps of hemosiderin corresponding with the phagocytosed blood pigment noted in the stained sections. The spleen also contains large clumps of hemosiderin, some of which are free and some phagocytosed.

Blood changes.—There is a progressive anemia, in some animals the blood becoming very thin and pale. Autoagglutination of red blood cells can always be detected. A lymphocytosis is constant. The blood pictures in several animals are shown in the following table:

MULE 239.	March 17.	March 23.
Polymorphonuclear leucocytes.....	60	49
Small lymphocytes.....	18	19
Large mononuclear leucocytes.....	18	25
Eosinophiles.....	4	7
	April 4.	July 16.
Red blood cells	2,584,000	1,672,000
Leucocytes	12,700	37,200
MULE 520.	March 18.	
Polymorphonuclear leucocytes.....	26	
Small lymphocytes.....	39	
Large mononuclear leucocytes.....	34	
Myelocytes	1	

MULE 228.	March 17.	March 23.
Polymorphonuclear leucocytes.....	24	19.3
Small lymphocytes.....	31	31.5
Large mononuclear leucocytes	43	45.5
Eosinophiles	2	3.7

MULE 263.	March 17.
Polymorphonuclear leucocytes.....	44
Small lymphocytes.....	25
Large mononuclear leucocytes.....	31

MULE 257.	March 26.
Polymorphonuclear leucocytes.....	45
Small lymphocytes.....	32
Large mononuclear leucocytes.....	23

MULE 487.	March 11.	March 14.
Polymorphonuclear leucocytes.....	31	51
Small lymphocytes.....	45	36
Large mononuclear leucocytes.....	22	13
Mast cell.....	1	
Large mononuclear containing a phagocyted red blood cell	1	

Auto-agglutination of the red blood cells occurs in many animals. It appears in experimentally infected mules and horses a day or two before trypanosomes are demonstrable in the coverslip preparation, and it lasts until death. In guinea-pigs after several weeks the phenomenon disappears and it disappears in animals that have recovered from the infection (agouti, cats). As observed by Yorke with *Tr. gambiense* the agglutinins are iso, auto, and hetero in nature; for example, the blood serum of an infected mule agglutinated the washed corpuscles of normal rats, cat, and man. The blood serum of naturally infected native horses agglutinated the washed corpuscles of a normal horse, an infected mule, a normal man and a normal and infected guinea-pig, and on the other hand the washed corpuscles of an infected native horse were agglutinated by his own serum and by that of an infected mule. These reactions, however, do not appear to be constant, possibly on account of inconstant amount of agglutinins, but it would seem possible to diagnose latent trypanosomiasis in mules and horses and

other animals by noting whether the blood serum of a suspected animal contained notable amounts of agglutinins. In point of fact, this has occurred to me, for while working with the blood of Mule 318 infected with an avirulent strain of *Tr. hippicum* I observed that the blood serum agglutinated the washed corpuscles of normal rat, cat, and man. This mule had been treated with arsenic after being experimentally infected and trypanosomes at this time could not be demonstrated in the peripheral blood. The agglutinating power of the blood serum made me suspect that the animal had a latent infection and this was true, for upon inoculating a guinea-pig and white rats with large amounts of blood the mule was demonstrated to be infected by the appearance of trypanosomes in the blood of all sub-inoculated animals. It would appear from this observation that there is a possibility of the existence of latent infections in some animals apparently cured by various medicaments.

Examination of films from spleen, marrow, and liver of infected animals.— Films show to better advantage than sections the presence and morphology of trypanosomes. Obsolescent trypanosomes are encountered in rich infections in smaller mammals. Many of them have been phagocyted. In the liver and spleen phagocyted trypanosomes, blood pigment, and red blood cells are seen, the pigment being more constantly found in large amounts in horses and mules only. Phagocyted trypanosomes are better seen in preparations of the smaller mammals which usually show a higher degree of infection. In horses and mules the hepatic cells frequently contain numerous, round, dark colored granules, not staining exactly like bile, but undoubtedly closely related to it. They are definitely not hemosiderin. Trypanosomes have never been detected in films. In the spleen there is a considerable amount of blood pigment in large clumps, frequently it is refractile and stains red, brown or green, with polychrome stains. When it has lost its refractility its color is a very dark brown or black. When it has recently been phagocyted it resembles obsolescent red blood cells.

In the coat the hepatic cells contain much bronze pigment, while the endothelial cells contain masses of very dark colored hemosiderin. In guinea-pigs films from the liver contain many trypanosomes in all stages of decline, frequently they are agglutinated and usually stain somewhat poorly. Phagocytosis of trypanosome residues by very large mononuclear cells is seen. From the staining reactions it would appear that the trypanosomes have become obsolescent before phagocytosis had occurred. The engulfed trypanosome residues consist of little more than a nucleus and stain either deeply and homogeneously or consist of two bodies, the trophonucleus and kinetonucleus, or what appears to be a reduction of the nucleus. No blood pigment is detected. In the spleen large mononuclear phagocytes have engulfed trypanosomes residues, red blood cells and other undetermined bodies, probably basophilic red blood cells. There are also many free trypanosomes and residues. In the liver of a rat (*M. alexandrinus*) trypanosome residues had been phagocyted by polymorphonuclear leucocytes, as well as by mononuclear forms. In the liver of mice, besides some phagocytosis of trypanosome residues, there are masses of trypanosomes in which eight or twelve trypanosomes had become fused, the chromatin filaments had disappeared, but the tropho- and kineto- nuclei were quite distinct. In the spleen many free and phagocyted trypanosomes were seen in both polymorphonuclear and mononuclear cells. The trypanosome residues are frequently in two unequal parts due to an unequal splitting of the nucleus. When the trypanosomal residues have resulted from the fusion of several trypanosomes, the kinetonucleus is seen to have moved up close beside the trophonucleus.

DISCUSSION AND SUMMARY.

Trypanosomiasis (Murrina) is an intoxication resulting in cellular degeneration and necrosis. The continuity of endothelium is injured or destroyed and as a consequence effusions and edema and terminal ecchymoses arise. The toxic

agents elicit certain reactions in the host; namely, lymphocytosis, auto-hemagglutination, phagocytosis of erythrocytes and trypanosomes, hyperplasia of the spleen, bone marrow and lymph nodes, and cellular exudations in the kidney, liver, and elsewhere. Korke¹⁴ believes "that the toxic product of trypanosomes damages the osmotic membranes and increases their permeability to the tissue fluids, whereby the blood plasma is diluted, and that the dilute nature of the toxic blood plasma facilitates the onset of edema." On the other hand, this may be explained by the efforts of the host to destroy or remove trypanosomes which result in the elaboration of substances, either toxic of themselves or which in combination with trypanosome products are destructive to the hosts own cells.

The extensive terminal ecchymoses and petechiæ on the epicardium, endocardium, pleura, peritoneum, nasal and ocular conjunctivæ, renal lymph nodes and kidneys, are certainly due to the destruction of the continuity of the endothelium and knowing how large a part these endothelial cells play in phagocytosis in this infection, it may be that the endothelium in places is destroyed through some cytolytic mechanism closely related to the intracellular digestion following phagocytosis.

The localized edema, the effusions, and the glomerulitis would seem to be related to this disturbance of the endothelium.

The degree of splenic hyperplasia varies inversely with the size of the infected animal, being greatest in the smaller rodents, and is directly proportional to the anemia and to the degree of infection—the number of trypanosomes per cubic millimeter.

The mechanism of the removal of defunct red blood cells is more evident in the mules, for blood pigment is not so much in evidence in the smaller mammals in which the erythrocytes would seem to be more rapidly destroyed.

The hepatic necroses and the collections of lymphoid cells in the liver and kidneys are of special interest. There are two types of necrosis: First, the large, central zone areas,

in which the hepatic cells have undergone a hyaline, granular or fatty change, and in which there is usually no leucocytic exudation. Second, the smaller areas found in the intermediate and peripheral zones in which there is a polymorphonuclear and mononuclear leucocytic exudation replacing the parenchyma. Each type of necrosis suggests a different etiological factor. Again, in this infection there is a very marked lymphocytosis and in many of the areas of necrosis the cells may be entirely lymphoid in type or preponderatingly so. This preponderance of lymphoid phagocytosis, as in malaria and typhoid fever, requires further investigation.

The collections of cells in the liver, which have been studied in several forms of trypanosomiasis by Pettit, and similar collections in the kidneys, are of the polyblast and lymphocyte type, and they can be seen in many locations emerging from the arterioles, capillaries, and sinusoids, having come originally from the spleen, in which organ they may be seen on the peripheries of the Malpighian bodies. These cells probably come from the lymph nodes and bone marrow as well. The lesions in the kidneys are very much like those described by Councilman,¹⁶ who, in discussing the cause of the focal and mononuclear character of the lesions in his cases of acute interstitial nephritis in scarlatina, says: "There is some ground for believing that the physical conditions of the circulation may have something to do with their accumulation in the vessels in certain places. It is also possible that in the interstitial foci there may be soluble substances which exert a positive chemotaxis for them . . . the explanation of the foci cannot be found in primary focal degeneration of the epithelium — for polymorphonuclear leucocytes and not plasma cells are attracted by degenerated tissue."

Himmel¹⁶ believes that the intracellular digestion of bacteria is performed in the presence of an acid, and Opie⁷ has shown that enzyme contained in the leucocytes of an inflammatory exudate are capable of digesting proteid, both in an alkaline and in an acid medium, though their activity is

greater in the former. He also states that it is not improbable that the enzyme of the mononuclear phagocytes is closely related to, if not identical with, the autolytic enzyme which is contained in various parenchymatous organs.

The mononuclear leucocytes have their origin in lymphoid tissue; they phagocyte red blood cells, various other leucocytes, trypanosomes, malarial plasmodia, and spirochetes. They have an intracellular digestive mechanism and a digestive enzyme known as lympho-protase. The polymorphonuclear leucocytes have their origin in the bone marrow. They phagocyte bacteria and are especially concerned in inflammatory changes following bacterial infections. They are also concerned in the hepatic necroses of eclampsia. The digestive enzyme is leuco-protase. Considering these observations of Opie's the pronounced lymphocytosis in the peripheral blood and visceral blood channels, the marked predominance of mononuclear cells in the hepatic necroses, particularly in the peripheral zones in certain animals, the predominance of mononuclear cells in the areas of interstitial nephritis, and in the iris ciliary body and areas of dermatitis and orchitis; the hyperplasia of mononuclear cells in the spleen, lymph nodes, and bone marrow with phagocytosis of red blood cells in same, and the proliferation of mononuclear cells in the secondary deposits and in the blood stream, suggest that the toxic agents of this infection specifically attract and stimulate the production of cells of the mononuclear type.

The cellular collections in the liver necroses are phagocytic in character and represent efforts to remove normal or degenerate hepatic cells. Megakaryocytes excepting in the sinusoids of the liver of the raccoon were not detected in the liver of any of the infected animals. On account of the active proliferation by karyokinesis in these portal collections of cells, as had been previously noted by Pettit, these cells in the kidney and liver act as temporary "field outfits" in contradistinction to "base supplies" and that they are in effect minute lymphoid nodes from which local supplies of

cells and antagonistic substances are sent out to take part in phagocytosis and the mechanism of defence.

The blood destruction and the stimulation of the blood-forming organs leads to hyperplasia of the yellow bone marrow and to an interesting picture in the spleen of the coati, guinea-pig, monkey (*Nyctipithicus*), rat, and mouse. The spleens of the coati, guinea-pig, rat, and mouse were found normally to contain a few megakaryocytes and nucleated red cells. Eosinophiles were absent. Yet, when these animals were infected with *Tr. hippicum* the spleen, particularly that of the coati, took on to an intense degree the picture of red bone marrow, in that the megakaryocytes were greatly increased in number, and eosinophilic myelocytes and polymorphonuclear leucocytes, as well as nucleated red cells, were seen. In this animal it would appear that the latent myelopoietic function of the spleen had been greatly augmented. The interstitial collections of cells in the kidney of the raccoon contained here and there some clumps of eosinophilic polymorphonuclear leucocytes, but no megakaryocytes.

In other mammals it was noted that when megakaryocytes normally were not seen in the spleen, the trypanosome infection did not provoke their appearance in any other location than the marrow of long bones. It is not uncommon to encounter megakaryocytes in certain types of anemia in man. They are normally found in the liver of the human embryo at the third and fourth month, and Bunting¹⁸ has shown that in provoked anemia of the rabbit the spleen takes on hemopoietic functions usually belonging to the marrow. If it is true that the megakaryocyte is the parent blood cell, then its appearance in its embryotic sites in the liver and spleen is an expression of the degree of anemia and of blood regeneration and of the necessity for the proliferation of blood cells in regions outside of the overtaxed marrow.

One of the most characteristic features of fatal trypanosomiasis is the phenomenon of auto-agglutination of erythrocytes. In the experimentally produced disease in mules it frequently appeared before trypanosomes could be demonstrated in the peripheral blood. Besides this agglutination

there is phagocytosis of erythrocytes in the peripheral blood stream, liver sinusoids, lymphoid tissue, and spleen. The erythrocytes are phagocyted by large mononuclear cells which become swollen and in some cases appear to occlude the sinusoids. Hyaline thrombi, however, were never seen. Two factors, then, are present which, as has been shown by Pearce,¹⁹ Mallory,²⁰ and Flexner, cause hepatic necroses — hemagglutinins and swollen endothelial cells. The true cause of these necroses, however, would seem to be that of which the swollen endothelium and hemagglutination are secondary manifestations and it will probably be found to depend on specific autolytic processes, and the phagocytosis of autolized cells.

We note in this trypanosomal disease that the lesions conform to those of other trypanosomal infections. The lesions encountered in mules and horses were constant, though such features as edema, emaciation, and ecchymoses varied with the stage of the disease. The gross lesions characteristic of the disease in equines are hemorrhagic petechiæ or ecchymoses of the pleura, pericardium, nasal and conjunctival mucosæ, peritoneum, renal cortex, and capsule of the spleen. There are hemorrhages of larger size in the endocardium, epicardium, and renal lymph nodes. Emaciation and anemia are usually constant, though in the fulminating type of the disease the former may not be present. Effusions or localized areas of edema of the sheath or hock, and particularly a longitudinal strip of the lower belly wall, are seen. There is slight splenic enlargement and myeloid hyperplasia of the yellow marrow.

In smaller mammals hemorrhages are not nearly so common; in fact, they are generally absent. Splenic enlargement becomes more noticeable and constant in the smaller animals. A remarkable lesion was studied in the dog, raccoon, and monkey — *Nyctipithicus* — acute iridocyclitis. Rabbits always presented a dermatitis of the ears and muzzle and a marginal blepharitis. Ulceration of the scrotum was occasionally seen in guinea-pigs.

Histologically, the lesions are distinctive. Nephritis was

noted in horses and mules, but was different in type from that seen in the raccoon and monkeys. The cellular changes in the liver in the guinea-pig are constant for that animal. The features of the lesions in horses and mules are agglutination of red blood cells and their phagocytosis by endothelium and large mononuclear phagocytes in the liver sinusoids, lymph nodes, and splenic sinuses; and hepatic necroses of two types, hyaline and inflammatory. There is lymphocytosis. Acute glomerulitis and acute hemorrhagic nephritis are very constant, as are the various petechiæ and ecchymoses, in which there may be a polymorphonuclear or mononuclear leucocytic exudation. In the spleen the chief change noted is the very large amount of hemosiderin brought thither by large mononuclear phagocytes.

These changes occur with great rapidity in small rodents, such as the rat, and in these animals a great many trypanosomes are produced in proportion to the size of the animal in a short space of time, so that they are overwhelmed rapidly. One never encounters as many trypanosomes in equines as in rats, mice, and monkeys. Though equines are ultimately killed by the infection, the lesions presented are, excepting for the associated anemia, very acute in point of time — terminal.

The histological changes in smaller mammals are generally more extensive when similar in type than those of equines. The hepatic necroses in the rat are more extensive than those in any other animal examined excepting possibly the coati. The necroses are of two types, the hyaline and the inflammatory. The former are usually limited to the central zone, while the latter are distributed everywhere. The lymphoid infiltration in the peripheral zones near portal spaces was seen in the mouse, guinea-pig, *Nyctipithicus*, and the mule. Associated with these collections of cells there were necroses, polymorphonuclear leucocytic exudation and areas of hemorrhage, and they are analogous to those changes described by Roudsky²¹ in livers of mice infected with *Trypanosomal lewisi*.

The spleens show hyperplasia of all the lymphoid elements, some cellular necrosis and phagocytosis of pigment and of trypanosomes. In the coati and mouse there is a remarkable hyperplasia of megakaryocytes normally found in small numbers in these animals. In the kidneys of the smaller animals ecchymoses are uncommon, and the glomeruli are not involved. In the monkeys and raccoon there is a very striking acute interstitial nephritis, very much like that described by Councilman in scarlatina and diphtheria. In the mouse there is an exaggeration of the hepatic and splenic necroses seen in the guinea-pig. The skin lesions and those of the iris show microscopic changes of the type characteristic of trypanosomiasis.

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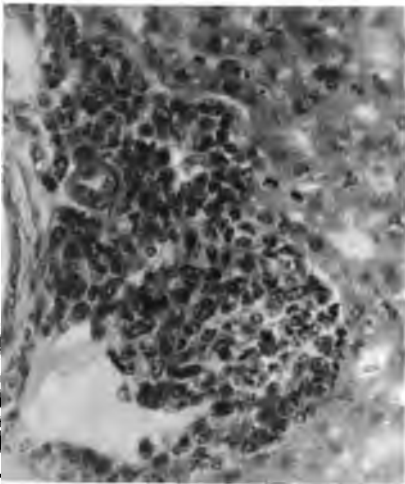
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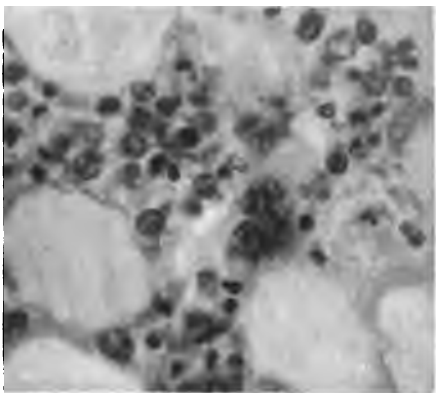
DESCRIPTION OF PLATES XIII. AND XIV.

- FIG. 1. — Liver of guinea-pig. Focal peripheral zone necrosis.
- FIG. 2. — Femur marrow of horse, showing hyperplasia and a megakaryocyte.
- FIG. 3. — Spleen of coati, showing hyperplasia and a megakaryocyte.
- FIG. 4. — Spleen of coati — normal spleen.
- FIG. 5. — Spleen of coati infected with *Tr. hippicum*, showing the intense lymphoid hyperplasia. Same magnification as section of normal spleen.
- FIG. 6. — Liver of rat, showing extensive necroses.
- FIG. 7. — Mule No. 239: Spleen with capsular ecchymoses.
- FIG. 8. — Mule No. 359: Spleen. Phagocytosis of erythrocytes and large clumps of blood pigment.
- FIG. 8. — Mule No. 359: Liver. Clumps of bronze colored pigment in the sinusoids and leucocytosis.
- FIG. 10. — Mule No. 382: Kidney. Acute glomerulitis.
- FIG. 11. — Mule No. 531: Epicardial fat, showing petechiæ.

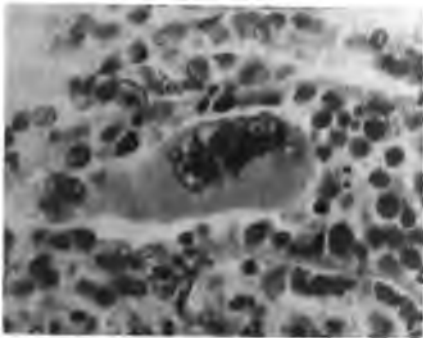
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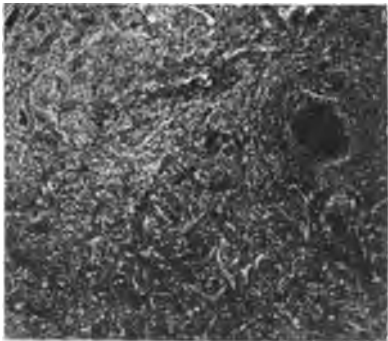
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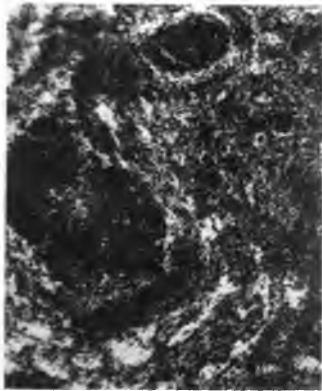
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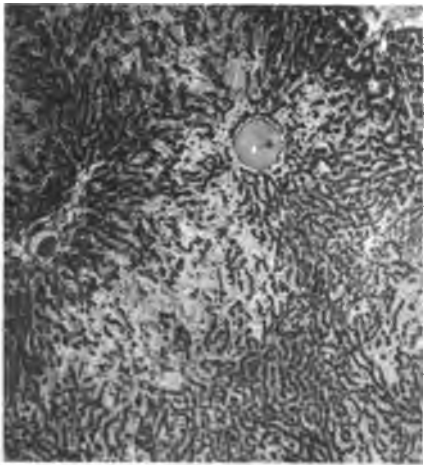
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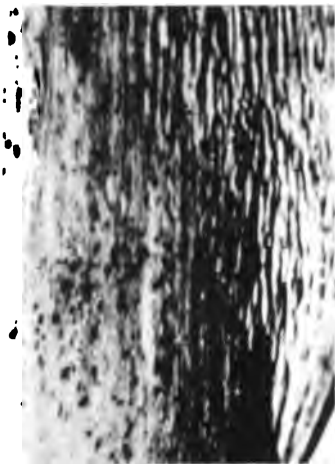
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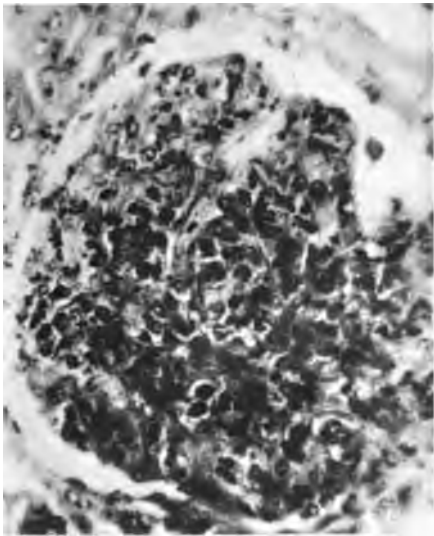
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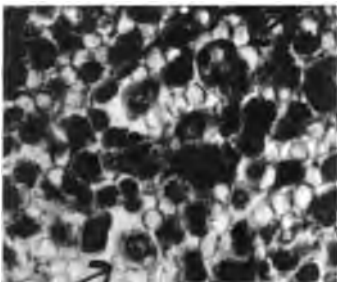
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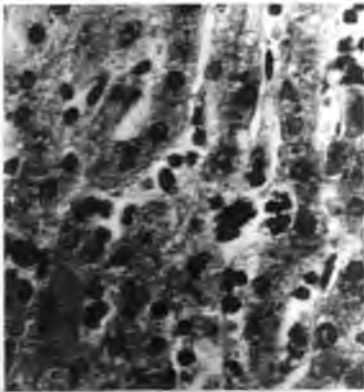
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SPONTANEOUS NEPHRITIS IN WILD RATS.*

W. OPHÜLS AND GEO. W. MCCOY.

(From the Laboratory of the U.S. Public Health and Marine Hospital Service at San Francisco and from the Pathological Laboratory of Leland Stanford, Jr., University.)

So far as we have been able to determine there are only two references to this very interesting condition in medical literature. The first one is a short note by one of us (McCoy) in the Public Health Reports of the U.S. Public Health and Marine Hospital Service, 1908, XXIII., 1365, where a number of pathological conditions, found in wild rats at the Federal Laboratory at San Francisco, are described. It is here stated: "It was a matter of considerable surprise to note that a number of wild rats are the victims of marked parenchymatous nephritis. . . . The kidneys are larger than the size of a rat would lead one to suspect, usually yellowish-brown or slate-colored, and are very friable. . . . Cysts as large as a pea, or even much larger, are not rare and small cysts up to about one millimeter in diameter are very common. . . . Microscopic examination shows the epithelium to be largely desquamated. The nuclei of the cells are lost or take the stain poorly. A few small cysts are seen scattered through the organ, and they may be quite numerous directly beneath the capsule. The process does not appear to affect the large collecting tubules and the glomeruli are generally but little altered. In some areas there may be marked increase in connective tissue."

In 1911 the other one of us (Ophüls) reported on one of these rats to the Society for Experimental Biology and Medicine (Proceedings, 1911, VIII., 77). This case was of especial interest as the renal lesions in it were associated with a moderate but apparently quite distinct cardiac hypertrophy and with a very marked general anasarca.

* Received for publication March 20, 1912.

The following data are the result of further observations and are based on the careful gross and microscopic examinations of forty additional cases.

The disease is found in San Francisco and in Oakland in the three types of wild rats: *Mus norvegicus*, *Mus rattus*, and *Mus alexandrinus*, both in the male and female. Because of the greater prevalence of *Mus norvegicus* it is most commonly observed in that species. Usually it is found in adult animals, but sometimes in the young. It is remarkably common. It occurs in well-marked form, readily recognizable to the naked eye, about twice in one hundred rats examined. What is still more remarkable is that the condition showed exactly the same type in all cases, perhaps with one or two exceptions. If other types of chronic nephritis occur in wild rats they are certainly quite rare.

The gross appearance of the diseased kidneys is very characteristic. The normal size of the kidneys of rats is about 25 x 10 x 10 millimeters. The affected organs are usually somewhat enlarged. The largest kidney found measured 25 x 20 x 15 millimeters. The capsule strips easily, is either entirely unchanged or, rarely, somewhat thickened. The color of the kidney is often distinctly more yellowish-brown than normal, sometimes more or less coppery. The surface is finely granular, at least when the process is sufficiently far advanced. The cortical tissue is usually moist, soft, and spongy. The pyramid is also moist, distinctly hyperemic, whereas the cortex is pale. The most characteristic feature of the process is the development of innumerable small cysts in the cortex, some of which may reach considerable size up to one centimeter in diameter or even larger. These cysts are either filled with thin clear liquid or the material contained in them is of white color and opaque. Occasionally small hemorrhages are observed. In a few cases the renal pelvis was considerably distended and filled with gelatinous material. In the end the kidneys shrink considerably, sometimes to one-half their normal size.

The most important lesion microscopically is a very

marked degeneration and eventually necrosis of the epithelial lining of the large majority of the uriniferous tubules with the exception of the large ducts. The epithelial cells are swollen, more or less vacuolated, full of minute fat droplets and eventually they completely lose their nuclear staining. The necrotic epithelial cells become detached from the walls of the tubules and fill the lumen. This intense degenerative process is associated quite early with an equally vigorous peculiar proliferation of those epithelial cells which retain their vitality, in such a way that in some tubules one finds the epithelium completely destroyed; in others it appears to be in a remarkably active state of proliferation.

The earlier stages of the process are well shown (Fig. 1) where many tubules show the extensive epithelial destruction and others marked evidences of epithelial proliferation. (See a group of tubules near the center of the picture.) The newly-formed epithelial cells are very large, two or three times normal size and the nuclei in them are often of immense size, regular giant nuclei, which stain very deeply. (These proliferative processes in the renal epithelium resemble very much those described by Thorel in man, but they are of a much greater intensity.) This proliferative process is also shown very clearly in Fig. 2, which is taken at the same magnification as Fig. 1. The epithelial degeneration is still apparent in some of the tubules, in others a tremendous proliferation of the epithelium has set in which leads to a very marked enlargement of the tubules. One also recognizes in this latter figure that the epithelial proliferation is apt to extend into the capsules of the glomeruli, as a result of which the outer layer of the capsule becomes lined with a more or less continuous row of cuboid or almost columnar cells (see glomerulus in center of Fig. 2). The newly formed epithelial cells also, in their turn, may undergo degeneration and necrosis to such an extent that often the dilated tubules and capsules of the glomeruli are filled with granular detritus in which no intact cells are left.

Directly associated with this proliferation of the epithelium in the tubules is the development of cysts which is the

most characteristic feature of the process. In Fig. 2 one can easily trace the development of these cysts from the dilated tubules. As the epithelium proliferates the tubules gradually enlarge until the cysts are fully formed. These cysts are filled with the detritus from the degenerating epithelium and some fluid, but the enlargement of them is quite evidently not due to pressure from the accumulating contents because there is no flattening of the epithelial lining, nor from retraction of new formed connective tissue around them, because at this stage they frequently do not show any development of new connective tissue in their surroundings.

In these later stages one observes in the degenerated epithelium the formation of large hyaline droplets which often reach the size of a red blood corpuscle or over. Some of the larger ones of these are shown free in the contents of the cysts in Fig. 2. They resemble somewhat the similar hyaline droplets observed in the epithelium of human cases of chronic parenchymatous nephritis. In rat nephritis they also stain deeply with eosin except when they have a color of their own, namely, a deep yellowish brown. The pigment which stains them in this way is evidently a derivative of the blood pigment. Once in a while one also finds some ordinary granules of hemosiderine.

In Fig. 3 there is represented a peculiar tumor-like proliferation of the epithelium which sometimes takes place in the larger cysts. In fact in some specimens small true adenomata like those which one so frequently encounters in human kidneys were observed.

Cast-formation is comparatively limited in this type of nephritis.

As shown in Fig. 1, the peculiar lesions in the epithelium are associated from the beginning with considerable cellular infiltration in the connective tissue. Eventually much dense fibrous tissue is formed in an irregular manner. In the end there may be so much destruction of the renal tissue by the degeneration of the epithelium and the proliferation and shrinkage of the connective tissue that the presence of the

glomeruli alone gives a clue that we are dealing with kidney tissue.

The glomeruli remain practically normal to the end except that the capsule may eventually be somewhat thickened.

In spite of the often very extensive destruction of renal tissue it is very uncommon to find edema or evidence of cardiac hypertrophy. In addition to the one case previously reported there is only one more in this new series of forty cases in which there is noted a moderate hypertrophy of the heart and large effusions into the pleural cavities, but no general edema. It is of some interest that in this case an old thrombus was found in the left auricular appendage which is plain evidence of considerable general circulatory disturbance. In a third case there was general anasarca and double hydrothorax but a normal heart.

No gross or microscopic evidence of disease of the blood vessels was found in any case.

These observations are renewed evidence of the rather loose association of severe renal lesion on the one hand and cardio-vascular disease and edema formation on the other, which was very evident in a series of human cases of chronic nephritis recently studied by one of us (Ophüls).

The close relation of these chronic proliferating inflammations to true tumor-formation is again brought out very plainly.

The cyst formation observed is very interesting from the standpoint of general pathology, as we have to deal here with cysts the origin of which can be referred solely to epithelial proliferation, a possibility which is doubted by some of our best authorities.

The etiology of the condition is entirely obscure. It is true that for years the rats, in San Francisco especially, have been poisoned with phosphorus and arsenic, but this has not happened for some time, quite apart from the fact that all experimental evidence at hand would make it rather unlikely that these poisons could produce such very marked and progressive renal lesions.

The peculiar proliferation of the epithelium and the tendency to the formation of cysts might suggest the presence of some protozoön parasite, like the coccidium oviforme or the coccidia described by Smith and Johnson¹ or by Tyzzer.² Careful examination, however, of fresh specimens or of smears stained carefully and intensely with Wright's stain or of sections stained with various methods fail to reveal anything in the nature of a protozoön, or of any parasite.

It would almost appear as if this unusual tendency to proliferation and cyst-formation was something inherent in the tissues of the rat, and in order to test this supposition some experiments on white rats were performed with the use of uranium nitrate.

It is a well-established fact that subcutaneous injections of uranium nitrate in proper doses and at proper intervals produce a typical chronic nephritis in all animals so far tested.³

Two white rats received a few doses of one milligram of uranium nitrate at intervals of several weeks and both developed the typical uranium nephritis: degeneration and necrosis of the epithelium and later cellular infiltration and proliferation in the interstitial tissue.

In these animals also the marked power of regeneration and tendency to excessive proliferation of the renal epithelium was very evident as shown in Fig. 4. The picture shows several large tubules with unusually large cells with large giant nuclei, just like those which were observed in the spontaneous nephritis of wild rats, and the tendency to cyst-formation is also very evident.

Control white rats were entirely free from all lesions, and the lesions which developed in the animals under experimentation were proportional in severity and extent to the time which the treatment had lasted.

It would seem, therefore, that this peculiar characteristic of the lesions is the result of a property inherent in the kidneys of rats. Here we have, then, an interesting example of a specific feature of a disease which does not depend so much on its cause, but on the nature of the animal.

As indicated above, similar proliferative changes occur in other animals under similar conditions, but so far as we know they never assume these extraordinary proportions.

In conclusion we may summarize the results of the investigation in the following way:

1. Wild rats very frequently (at least in two per cent of all cases examined) suffer from a peculiar type of chronic nephritis, one of the characteristic features of which is a very marked tendency to epithelial proliferation and to cyst-formation.

2. A condition very similar in all respects to this spontaneous disease may be produced experimentally in white rats by subcutaneous injection of uranium nitrate.

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3. See E. C. Dickson. A report on experimental production of chronic nephritis in animals by the use of uranium nitrate. *Archives of Int. Med.*, 1909, iii, 375. An additional report will appear shortly.

EXPLANATION OF PLATE XV.

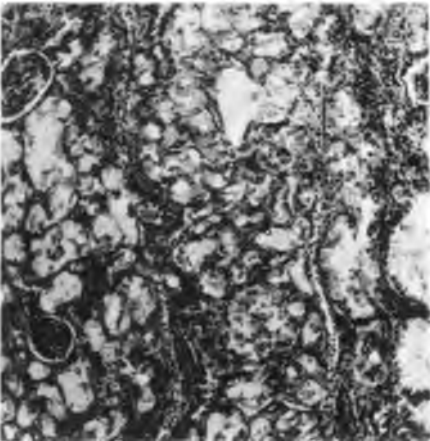
FIG. 1. — Magnification 80 x. Early spontaneous nephritis in wild rat showing extensive epithelial degeneration; also marked epithelial proliferation and cyst-formation, cellular infiltration of the interstitial tissue.

FIG. 2. — Magnification 80 x. Similar but more advanced lesions, extension of epithelial proliferation into glomerulus.

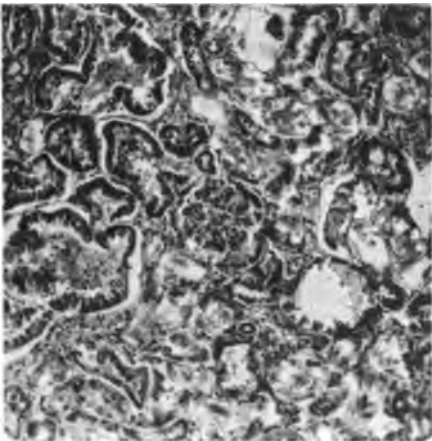
FIG. 3. — Magnification 80 x. Adenomatous growth into cyst in spontaneous nephritis.

FIG. 4. — Magnification 100 x. Reproduction of spontaneous nephritis of wild rat by subcutaneous injections of uranium nitrate in white rat.

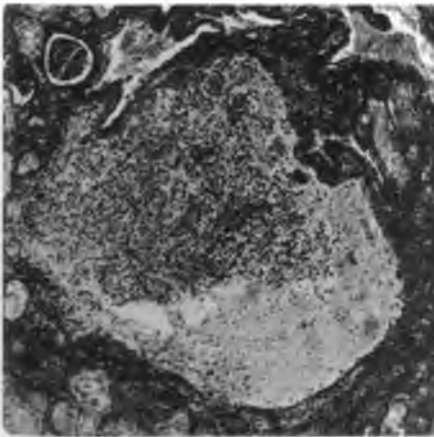
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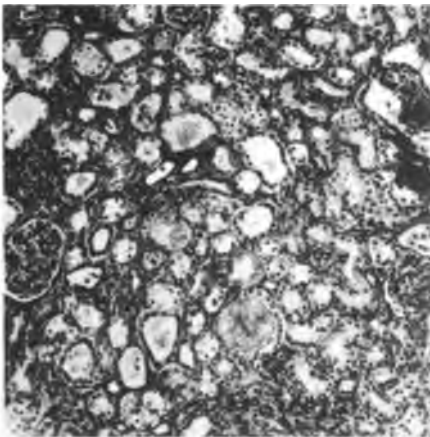
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Ophüls

Nephritis

THE RELATION OF ANIMAL FAT TO TUBERCLE BACILLUS
FAT.*

(A SUGGESTED EXPLANATION OF THE APICAL LESIONS IN
MAN AND CAUDAL LOBE LESION IN CATTLE.)

(Preliminary Communication.)

WM. CHARLES WHITE, M.D.

AND

A. MARION GAMMON.

(From the R. B. Mellon Laboratory of the Tuberculosis League of Pittsburgh.)

The relation between the fat stored in the animal body and the fat in the tubercle bacillus growing in this animal body seems at first glance a very indefinite one; and yet an attempt to establish such a relation yields many points of interest, the correlation of which establishes a connection between the two which is apparently very close.

The storage of fat in bodies that are the seat of tubercular infection is usually the basis of a favorable prognosis. While this is accompanied by a recession of other symptoms of tuberculosis such as fever, tiredness, sputum, and lung signs, still it is the storage of fat which is the striking feature of improvement both to patient and physician. This storage of fat, too, is the main object of the tripod of rest, fresh air, and feeding which forms the foundation of our treatment in this disease.

On the other hand, in bodies which are the seat of a tubercular infection which is gaining the upper hand, no feature is more striking than the rapid consumption of the fat which was previously stored in the fat reservoirs of the body. This consumption of fat is in most cases greater in amount than the accompanying fever would explain.

One of the problems connected with this relation of fat to tubercular infection is associated with the varied composition of the fat occurring in different animals in which the

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disease occurs. For example, the fat of humans after infancy is largely¹ composed of the glycerides of palmitic and oleic acids. The fat of infants,² on the other hand, has a larger percentage of the glycerides of stearic acid and in this way more closely approximates the fat of cattle which has a larger percentage of the glycerides of stearic acid.³ The fat of fishes has a larger percentage of the glycerides of oleic acid.⁴

It occurred to us that this wide variation in the composition of the fats of different animals may in some way be closely connected with the variation in the different types of tubercle bacillus, and that, as an instance of this, the stearic acid content of infant's fat, approximating as it does cattle fat, may contain the nucleus of the greater susceptibility of infants to the bovine bacillus.

This conception seems more likely to contain some truth from the various studies which have been carried out on the fat content of the various types of tubercle bacillus. The tubercle bacillus has a very large content of fatty substance; this fat content varies in character and amount under varying conditions of growth and origin. Ruppel⁵ found the fat content varied with the age of the culture from eight to twenty-six per cent. De Schweinitz and Dorset⁶ found that the total extracts from the tubercle bacillus of different origins using three extractives, ether, alcohol, and chloroform, to be as follows:

Bovine bacilli,	26.32%	Avian bacilli,	30.65%
Swine "	20.59%	Attenuated human bacilli,	37.41%
Horse "	31.76%	Virulent " "	28.03%

In this analysis the most striking fact, and this we will refer to later, is the high percentage of fat extracted from the attenuated human organism. Aronson⁷ found that with a low percentage of glycerine the wax content of the tubercle bacillus grown on media was much reduced. These studies seem to be in some way associated with the different behavior of the human and bovine bacillus grown on glycerine broth which Theobald Smith⁸ has pointed out. It is

possible that the use of glycerine by the tubercle bacillus is associated with its relation to fat since the glycerine forms so large a factor in fat composition. This is no new observation and is mentioned here only to point out, in connection with the present work, that the close resemblance which Smith found to exist between the bovine and avian bacillus may be associated in their origin from grain-eating animals whose fat content is largely composed of the glycerides of stearic acid.

We proposed a year ago to attempt to work out some of the problems connected with the relation of the fats of the tubercle bacillus to body fat both from a biological and cultural standpoint. In the present paper we will confine ourselves to certain cultural characteristics which we have found and to the bearing of these on certain anatomical conditions found in man and cattle especially relating to the point of maximum attack in pulmonary tuberculosis of these two animals. Our work on the influence on tuberculous infection of various types of fats stored in the lungs before and during the period of infection and on the influence of the components of these fats such as glycerine and various fatty acids in the same connection we will report in later papers.

In choosing the various fats for this work many difficulties were encountered. It was desired, in the first place, to secure fats which were fluid enough to mix with culture media and yet which contained as high a percentage as possible of a single one of the five radicals with which we wish to deal (as the basis of most body fats), viz. : stearic acid, oleic acid, palmitic acid, and linolic and linoleic acids. With this object in view, we used olive oil for the oleic acid, palm oil for the palmitic acid, linseed oil for linolic and linoleic acids, and beef suet for the stearic acid. The latter was difficult to handle on account of its high melting point. We also used more complex fats such as butter and human fat. Our work is open to serious objections of which we are aware but, as with all new fields, we had to start with that knowledge which was available, and in later papers we will have corrected some at least of these difficulties.

It was found that in the test-tube certain fats had a decided influence in determining the rapidity of growth of the tubercle bacillus as well as its staining properties and virulence. To five per cent of glycerine agar tubes were added six per cent and twenty per cent of the following fats: Human fat, olive oil, butter, linseed oil, beef suet, paraffin, and palm oil. The human and beef fat were obtained at autopsy and rendered in the laboratory over a water bath. The tubes were then sterilized and after sterilization cooled; then when near the solidifying point, vigorously shaken, rapidly cooled on ice and slanted; in this way a uniform distribution of the fat in the glycerine agar was obtained. Five tubes of each fat glycerine agar and five tubes of plain glycerine agar as control were then inoculated with tubercle bacillus of human and a similar series with tubercle bacillus of bovine origin transferred from egg media. The tubercle bacilli used in these experiments were from strains H₃₇ and Bl Saranac Lake kindly furnished us by Dr. E. R. Baldwin.

The human bacillus grew best on the human fat agar and butter agar tubes. The growth on the olive oil agar tubes was not so abundant as on the control glycerine agar tubes, and the growth on the linseed oil agar tubes was very slight.

The bovine bacillus did not grow so abundantly as the human strain, but the growth on the human fat, olive oil, and butter tubes was much in advance of the control glycerine agar tubes. The growth on the linseed oil tubes was scarcely noticeable at the end of six weeks.

The beef fat in tubes did not stimulate the growth of either organism, although both grew slightly and were cultivated again on egg media after two months.

The palm oil tubes were not successful on account of the difficulty in securing uniform distribution of the fat in the agar. Both human and bovine organisms grew on the agar between the fat-containing areas of the slant but not on the palm oil.

On paraffin the organism grew after eight to ten weeks with a dry brownish appearance in the growth.

The difference between the growth of the human tubercle

bacillus on the five tubes of human fat glycerine agar and the growth on the five tubes of plain glycerine agar was very striking and was the same on all tubes, being one hundred times more abundant on the former tubes. The growth on the fat-containing tubes presented a peculiar oily appearance to the eye.

Smears were made after six weeks from the various tubes, stained in carbol fuchsin and the time of decolorization determined by immersing for various times in four per cent nitric acid in equal parts of acetone and alcohol. It was found that at the end of six minutes the organisms from the plain glycerine agar tubes were almost completely decolorized, while after ten minutes the organisms grown on human fat, olive oil, and butter agar still retained their stain very evenly and well.

This we hope to show has some bearing on the fact noted by De Schweinitz and Dorset, that the fat content of the avirulent human type which they studied had a far greater fat content than the virulent form. The increased acid-fast character of those organisms grown on fat noted in our own work suggests a decrease in the virulence of the organism, but this aspect of the work must be carried much further before such a conception can be accepted.

After repeating these series of experiments a number of times and finding that the influence of fats of various sorts in the culture media had a constant and definite influence in increasing the rapidity and character of the growth of the tubercle bacillus in the test-tubes, we took the same fats and submitted them, for various periods of time, to the action of liver extract prepared after the method described by Kastle.¹⁰ We then took these mixtures of fat and liver extract and after sterilizing mixed them in various proportions with five per cent glycerine agar prepared as in the former unaltered fat experiments, and again inoculated five tubes of each mixture and five tubes of plain glycerine agar (used as control) with as nearly uniform quantities as possible of human and bovine tubercle bacillus.

The results of these experiments were in some instances

more striking than the pure fat experiments. The growth of the human tubercle bacillus on each liver-altered human fat-agar tube was four or five hundred times more luxuriant than on the plain glycerine agar. The growth on all the liver-altered olive oil and liver-altered linseed oil tubes was also more abundant than on the plain glycerine agar tubes.

The growth of the bovine bacillus was much more abundant on the liver-altered olive oil and linseed oil agar tubes than on the plain glycerine agar tubes, but on all three of these groups less than on the liver-altered human fat agar tubes.

The other fats were not included in this experiment, which has been repeated with the fats named above.

Explanation of apical lesions in man and caudal lesions in cattle. — The results of the use of these various fats and split products of these fats on the growth of the tubercle bacillus have suggested an explanation of:

1st. The apical lesions which are so common in tubercular pulmonary infections in man.

2d. The caudal lobe lesions which form the characteristic expression of tubercular pulmonic disease in cattle.

Having determined that the tubercle bacillus is able to use various fats and their liver-split products to its own advantage when grown on artificial media, it seemed possible to correlate this fact with the work of J. B. Leathes⁹ on the function of the liver in mobilizing and changing the fats already stored in the subcutaneous tissues in explaining why the favorite site of growth of the tubercle bacillus in man is the highest point in the lung. Leathes was able to show that the fats stored in the reservoirs of the animal were mobilized in the liver and here prepared for utilization by the other organs of the body which during their action burned the fat. This liver-altered fat is of necessity poured into the inferior vena cava and thence finds its way into the pulmonary artery and from here passes directly into the lung capillary system. If one studies the anatomy of the pulmonary artery, however,

one sees that before dividing into the right and left pulmonary branches it forms a great bay of blood bounded by a very elastic vessel wall. The blood in this portion of the pulmonary artery must move with comparative slowness, because of the short circuit which it makes in comparison with the long circuit made by the general aortic quota of blood. In this great bay the blood is loaded with fatty derivatives of low specific gravity compared with the whole blood. Owing to the slowness of the current due to the elasticity of the vessel and the short circuit to be traveled, these compounds have a chance to rise to the surface of the stream, so that the upper layer of blood in the pulmonary artery should have a much larger content of fatty compounds than the lower one. If one now follows the pulmonary blood stream in man it is readily seen that at the highest point of this main stream the vessel arises that supplies the apex of the upper lobe on either side. If the theory advanced be correct, this vessel, owing to the upright position in man, should be the vessel most laden with the fatty compounds of low specific gravity which are being poured into the pulmonary stream by the liver mechanism because these compounds, of necessity, rise rapidly to the highest level of the fluid bed in which they are traveling. This view, coupled with the results of our experimental work so far completed, which shows that the tubercle bacillus makes use of these compounds for its more abundant growth, seems a most reasonable explanation of its more prevalent development in the apex of the upper lobe. In other words, it is in the lung that the tubercle bacillus gets first pick, so to speak, of the fatty compounds which it needs for its development and receives the greatest supply of these at the apex of the upper lobe in animals that maintain an upright position.

Our next step was to determine on this basis where the highest point in the pulmonary blood stream of bovines lay, and by plaster injections we were able to determine that the same law as we have outlined for man if applied to bovines would carry the fatty compounds of low specific gravity to the apex of the caudal lobe. Having decided that this was

true we were sure that if the law held good we should find in this region the point of maximum attack in pulmonary tuberculosis in bovines. Not having the literature at hand to add the clinical proof to our theoretical conclusion, we wrote to Dr. Schroeder in Washington without mentioning what the trend of our work was and received the following letter in answer:

"The lungs of cattle have seven lobes as follows: two anterior; two median, somewhat larger than the anterior; two caudals, much larger than the median; and an azygos which is attached to the right anterior.

"The commonest location of tubercular lesions in bovine lungs is in the posterior dorsal portions of the large caudal lobes from the apices forward and upward. This region is dorsal from the main bronchus and rests under the ribs at or near their junction with the vertebræ.

"The only similarity I can trace between the seats of tuberculosis in human and bovine lungs, taking respective natural positions of human and bovine bodies into consideration, is that in both these species those portions of the lungs are more commonly affected which have the highest location."

It will be seen that the clinical data bears out fully our theoretical conclusion. We hope to be able in the near future to demonstrate that certain points concerning the pulmonary stream, which we have concluded should occur from a theoretical physical basis, occur in fact. If we are able to do this, we feel convinced that this offers the most reasonable explanation on a pure chemical and physical basis of the elective points of pulmonary tuberculous infection in man and bovines.

We were able, during the progress of this work, to go over it with Professor J. B. Leathes, of Toronto, who suggested that it would be wise to get rid of the extraneous substances contained in the liver extract by splitting off the fatty acids with sodium hydrate, then separating these with an excess of acid and, after washing, use these substances in the culture tube. In this way the experiment would deal with

simpler and purer compounds. This work is at present in progress and will be reported later.

M. P. Ravenel¹¹ also suggested that during some former work of his own he had noticed that the tubercle bacillus would grow on paraffin. This we have confirmed in our own work, but what use the tubercle bacillus makes of so foreign a fat as paraffin we are at a loss to understand.

The various aspects of the relation of fat added in abundance to the medium on which the tubercle bacillus is grown, to the virulence of infection in the body, we can at present barely touch upon. If fat and its split products make the tubercle bacillus grow, why, for instance, does increase of body fat indicate improvement? Some of our experiments suggest that bacilli overfed with fat in the test-tube become less virulent. For example:

- One milligram of tubercle bacilli grown on five per cent glycerine agar and human fat,
- One milligram of tubercle bacilli grown on five per cent glycerine agar and butter, and
- One milligram of tubercle bacilli grown on five per cent glycerine agar and olive oil

were injected into rabbits. The weights and first autopsies suggest as stated a diminished virulence in the fat-gorged organisms, but it is far too early to draw even tentative conclusions.

The above results further suggest an explanation of the susceptibility of certain diabetics to tuberculosis since "the occurrence of very large quantities of fat in the blood plasma seems to be a phenomenon peculiar to diabetes."¹²

We were not able to determine what we started out to prove, that such methods as we have employed offer a means of distinguishing the human and bovine types of organism; but the findings still suggest the possibility that in the relation of animal fat to tubercle bacilli there may be found some light on this question.

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THE CYANOTIC INDURATION OF THE KIDNEY.*

HORST OERTEL, M.D.

(*From the Russell Sage Institute of Pathology, New York.*)

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I. Introduction, anatomical findings, and views of older authors. — The intimate relation between diseases of the heart, circulatory disturbances, and certain functional and anatomical changes in the kidney had attracted attention almost from the time of Bright. Rayer¹ and Becquerel²

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had observed them in diseases of the heart, Devillier and Regnauld³ in pregnancy. They were described with care by Reinhardt⁴ in the first volume of the "Annalen der Charité" in 1850 and brought into proper etiological relation to long continued valvular disease of the heart, chronic bronchitis, and pregnancy. But like his predecessors he included them under the heading of Bright's disease. He considered the presence of albumen and casts (fibrin coagula) in the urine during life as evidence of an exudate, and therefore regarded the kidney changes as inflammatory. Reinhardt knew already that these changes in the kidney occurred particularly when certain circulatory disturbances like edema became manifest. While Becquerel had described a transitory albuminuria in heart diseases, Reinhardt emphasized its long duration in certain cases, and that even after lasting albuminuria the kidney showed only hyperemia and enlargement. He described them further as dense, firm, but microscopically he emphasized only the occurrence of fatty epithelium. Finally he considered as cause of the renal change a possible direct irritating influence of an abnormal composition of the blood in heart diseases. Eisenmann⁵ in 1852 also fully recognized the relation of venous stasis in the kidney to certain definite anatomical alterations. He described passive hyperemia as the result of a disturbed return flow of blood from the kidneys, which may extend from the tubules to the Malpighian corpuscles. This might be the result of local or general causes: heart diseases, respiratory diseases, convulsions. As regards the relation of pregnancy to Bright's disease and stasis in the kidney, Eisenmann, quite properly and against the views of Frerichs, pointed out that an impeded return flow of blood causes directly only a mechanical or passive stasis, and the passage of serum which results therefrom must be differentiated from that of exudative inflammation. Frerichs⁶ admitted this only partly, and believed that under the irritative influence of the blood mechanical stasis assumed the character of Bright's disease. But while Eisenmann did not deny this possibility he did not believe it necessary for the

production of albuminuria or uremia, nor did he believe it to be the rule. However, these views remained isolated and were not generally recognized. Rokitansky, in his great *Lehrbuch der pathologischen Anatomie*, published in 1855, devotes no space to these considerations, but only mentions hyperemia of the kidney in a general way. He did not differentiate between arterial and venous hyperemia, described only the vascular injection of the blood vessels and glomeruli and regarded it as antecedent of nephritis or Bright's disease.

It remained therefore for Traube⁷ to succeed in conclusively separating amidst considerable opposition the kidney of stasis from the inflammatory affections. This work began in 1856 and was constantly augmented by him by additional evidence and persistent discussions. In his first observations he gave an excellent description of the gross appearances of such a kidney, in which he emphasized particularly the vascular changes, absence of granulations, and the preservation of the relative volume of cortex and medulla. He sums up his conclusions as follows: "Experiments (referring to Ludwig and Goll) teach that such changes depend upon the diminished aortic pressure and increased venous pressure. If extending over a certain time they produce certain parenchymatous changes, the nature of which is still under discussion. I personally do not believe that we have to deal with the disease discovered by Bright, for it lacks the most important characteristics of this disease — fatty metamorphosis of epithelium, the leathery consistency of the parenchyma during its atrophy, the contraction of the cortex, and that coarse granulation of the surface which is so constantly to be observed during slowly progressing Bright's disease." He emphasized that neither albumen nor casts were necessarily the product of an inflammation, and that hyperemia and serous transudate were not always inflammatory in character " . . . "After all, it appears more natural to regard the kidney affection just described as similar to the nutmeg liver which occurs so frequently among heart cases under the same conditions." With regard

to the susceptibility of such kidneys to Bright's disease Traube also expressed himself: "Another question is whether this condition of venous hyperemia does not create a predisposition to Bright's disease. In spite of the difference in both affections this is evidently not impossible. The observations and statistics of Bergson⁸ and Chambers have lent some basis to this view, but they have failed to mention to what extent the valvular lesion injured the function of the heart, and whether Bright's disease or valvular disease were the primary disease." He then criticises their statistics and concludes: "Based on my own observations I venture to say that true *Morbus Brightii* as a sequence of such structural changes which diminish the activity of the heart is a rather rare phenomenon, that therefore the frequent occurrence of venous hyperemia of the parenchyma of the kidney creates no predisposition to *Morbus Brightii*. In subsequent communications Traube again emphasized that venous congestion of the kidneys was clinically and anatomically a lesion *sui generis*. He admitted later the occurrence of fatty changes in the tubular epithelium as the result of nutritive disturbances by a diminished arterial blood supply. But he persistently denied the termination of the process in the so-called granular atrophy of the kidney.

These views aroused considerable opposition. In a discussion following a paper by Traube read on Feb. 8, 1860, Leubuscher⁹ argued against extremely specialized and minutely detailed consequences based on certain facts as complicating diagnosis and practice. He believed a sharp line between the various kidney diseases as attempted by Traube to be practically impossible.

Rosenstein¹⁰ said that the effort to separate the conception of Bright's disease into several categories had already been initiated by Virchow. He did not agree with the idea that venous stasis in the kidney never led to atrophy. His own observations, as well as two by Frerichs, and one made by Reinhardt disproved it. Even in these cases the fatty degeneration of the epithelium initiated collapse and loss of tubules.

The subsequent discussion centered mainly around the characteristic anatomical features of inflammation. Traube concluded it by declaring: "That in his opinion it had to be sharply differentiated whether swelling and fatty changes were the result of inflammation or had resulted from other causes; alone they formed no criterion. The so-called parenchymatous inflammation or, better termed, parenchymatous process (after Beer) might occur in connection with many other lesions; but an inflammation produced not only swelling but proliferation." In the published report of these proceedings Traube has added a note in which he draws attention to the fact that he was really the first one to deny the existence of parenchymatous nephritis. These views of Traube, although classic in character, were slow in becoming generally recognized, for it remained the current view that valvular diseases of the heart led frequently, partly by stasis and partly by increased arterial pressure, to Bright's degeneration of the kidneys. Bamberger¹¹ particularly held that Traube's views would remain isolated. The examination of the kidneys in such cases, according to Bamberger, shows all those changes which are characteristic of Bright's disease, but they present mostly the early stages, particularly that of hyperemia, shown by an injection of the Malpighian corpuscles and a general cloudy appearance of the cortex with fatty degeneration of the epithelium and exudative casts into the tubules. Thus the condition may even progress to atrophy and granular contraction; he claimed to have observed the latter with certainty in some instances. Bamberger held that even Traube's own description stamped the process as an inflammatory disturbance of nutrition; a parenchymatous inflammation. The occurrence or lack of fatty changes, to which Traube and others had paid much attention, was considered of little consequence by Bamberger, as it was not constantly associated with inflammation. It was therefore Bamberger's opinion that a passive hyperemia might lead to inflammation as well as an active one, and he agreed with the statistics of Bergson, Chambers, and Willigk, which had been adversely criticised by Traube. The only

concession which Bamberger was willing to make was that in heart diseases the renal affection did not frequently progress beyond the first anatomical stage of Bright's disease.

Lecorché¹³ in 1875 still held that cyanotic induration of the kidney was a particular form of interstitial nephritis, while Kelsch¹² acknowledged its separation from Bright's disease in 1874. Lépine,¹⁴ in his monograph on the progress of kidney pathology (1884), has not included a consideration of venous cyanosis. However, the weight of opinion during the seventies had become decidedly in Traube's favor. Johnson,¹⁵ for instance, in his earlier work on the diseases of the kidney, published in 1852, rather opposed the idea of any changes in the kidney being the direct result of mechanical circulatory conditions as a consequence of heart or respiratory diseases, but had considered them complicated processes which resulted from changes in the blood due to imperfect oxygenation and accumulation of carbonic acid, and grouped them with Bright's disease. But he fully recognized and described chronic venous congestion in his lectures on Bright's disease in 1873 as an independent affection. He mentions the enlargement and induration of the kidney, the backward distention from the veins to the tubular and to the Malpighian capillaries; and the consequent transudation of serum through these capillaries. He described the Malpighian capillaries as sometimes ruptured by over distention, the urine is then blood tinged and contains blood casts. The ultimate result of long-continued congestion was in his opinion atrophy and contraction of the organ. The kidney becomes uneven, and, as the process goes on, finely granular. On microscopic examination some of the tubules may be found opaque with disintegrated epithelium and fibrin, some denuded and in various stages of atrophy and contraction. He states particularly that when atrophy of the kidney has been a result of passive congestion consequent on mechanical hindrance to the circulation, he has never found the muscular walls of the minute renal arteries

hypertrophied. The clinical pictures of the lesion and the urinary symptoms were then also given by him in detail.

Rosenstein,¹⁶ Klebs,¹⁷ Bartels,¹⁸ and Rindfleisch¹⁹ and all later authors recognized cyanosis of the kidney as an independent affection, but the histogenesis and ultimate fate of the kidney under these conditions remained under discussion, and still considerably confused. Bartels was decidedly against the inflammatory nature of the process. He held that in pure cases the characteristic accumulation of lymphoid elements in the interstices was absent, as well as inflammatory swelling of the cells, and that the fatty changes were part of the parenchymatous atrophy. The shallow cicatricial depressions on the surface he considered due to atrophy and wasting of certain portions of convoluted tubules with their glomeruli, and distinguished them from the deeper penetrating scars which were the results of embolic processes, frequently associated with venous hyperemia. He denied a diminution in size below that of the normal kidney, but emphasized that often the reverse is true, a venous congestion superadded to a contracted kidney. This combination, he held, had been the basis of much confusion.

Klebs gave this description of the cyanotic kidney: "The kidneys are enlarged, the surrounding capsule poor in fat; the dense fibrous capsule easily removed, after which the surface of the kidney appears entirely smooth and congested. The veins of Verhein are markedly dilated and engorged with blood. On section both parts of the organ are reddened, the medulla dark on account of strong engorgement of the straight vessels of the vasa recta, the veins of the medulla particularly engorged. The glomeruli are not prominent, but engorged. Microscopically veins and capillaries are markedly filled, but the epithelium of the tubules is usually not changed. The interstitial tissue is unusually dense, quantitatively somewhat increased and more prominent than normal. It shows a fibrillar structure." Klebs believed that edema and dilatation of lymphatics is infrequent, as the induration of the tissue had a tendency to prevent this. The transudation occurs therefore much more

abundantly in the capsule of the glomeruli and in other serous membranes. In recent cases he found around the glomerular capsule and the tubules lymphoid elements, which later led to cicatricial contraction and obliteration of tubules and glomeruli. Near the cortex such changes might lead to fine granular retraction. The condition may in his opinion exist for a long time without functional disturbances, but a slight decrease in arterial pressure may suffice to throw out albumen from the glomeruli. The blood, which is rich in carbonic acid and poor in oxygen, interferes with the nutrition of the epithelium, producing a granular degeneration, particularly in the convoluted tubules.

Rindfleisch considered that the induration in venous cyanosis consisted of a gradual swelling of the tissue. The elements enlarge, but the number remains the same. The intercellular tissue increases and assumes a fibrillar character. We deal, therefore, with a hyperplasia in a limited and strict sense, that is, a general increase of all the existing textures composing the connective tissue. Such a process, however, does not occur in any inflammation, but only in venous hyperemia and in contradistinction to inflammatory hyperplasia is a very general one. It leads therefore to a uniform enlargement of the organ. It depends upon better nutrition of the parts and throughout has the character of a homologous formation. Vessels and tubules are not changed by the connective tissue. The secretion contains at times albumen due to a periodic increase in blood contents of the kidney.

Cohnheim,²⁰ however, described in addition to the general engorgement of the blood vessels an ectasy of capillaries; and that the interstitial connective tissue showed not only cellular infiltration, but quantitative increase, while the epithelium became fatty only in the severest cases.

Runeberg²¹ found, that the interstitial tissue had lost its homogeneous form and changed to a more fibrillar structure. Usually it appeared slightly hypertrophied and at times considerably infiltrated with lymphoid cells. The membrana propria of the tubules has lost its glossy structure and

appears more firmly attached to the surrounding connective tissue. The epithelium at times shows more or less fatty degeneration, while the glomeruli show at least in the beginning no changes. Runeberg agrees with Rosenstein that later in the disease a number of glomeruli may become somewhat atrophic and thickened; but strongly doubts that any of these anatomical changes are sufficient to explain the functional disturbances in venous stasis, particularly the accompanying albuminuria. In support of this he cites a statement of Bartels that the same kidney, which for months has secreted small amounts of an albuminous urine of high specific gravity, immediately responds with secretion of an abundant albumen-free urine of light color and normal specific gravity when either natural or artificial events have reëstablished a normal aortic pressure and abolished the abnormal pressure in the venous system.

Ribbert²² found in a case of stasis swelling of the epithelium of the Malpighian capillaries and of the capsule, but no desquamation. Even after long continued stasis the tubules were found intact. The interstitial tissue was not broadened, but showed fine fat degeneration, as did also the epithelium of the glomerulus.

II. Modern anatomical observations and views. — Puricelli,²³ from Bollinger's laboratory, in 1886 gave a short review of previous findings and described the kidney changes in nine cases which he considered examples of the lesion. They showed broadening of the interstitial tissue almost exclusively in the medulla, colossal dilatation and engorgement of capillaries and small veins, also mostly confined to the medulla; granular degeneration of epithelium in the cortex and atrophic degeneration in the medulla; small cell infiltration in the cortex, particularly near the periphery and in the neighborhood of the glomeruli. He interpreted the thickening of the connective tissue as a non-inflammatory hyperplasia. The glomeruli in his cases were as a rule enlarged, cellular, with marked thickening of Bowman's capsule, and infiltrated with cellular elements, and there existed

slight retraction of the surface of the kidney by obliteration of glomeruli and their surrounding tissue. Puricelli's results have been generally adopted and handed down without much criticism. It is plain, however, on careful review of his evidence, that he fell into the errors of some other writers.

Cases 4, 6, and 7 were complicated by severe infections and there can be no doubt that the kidney showed not only the result of stasis but also nephritic lesions. Case 5 I should also regard as nephritis with stasis; Case 2 is complicated by arteriosclerosis; Case 8, however, appears to be a perfectly pure case (old left-sided valvular endocarditis) and its findings differ entirely from those of the others. There was no interstitial connective tissue thickening, the glomeruli and the connective tissue around the capsule with few exceptions normal, the vessels generally normal, no capillary ectasy. In the medulla the connective tissue was broadened and the blood vessels dilated. Case 9 also appears as pure stasis, although an autopsy protocol is missing. The cortex showed practically no changes, the medulla dilatation of capillaries and broadening of the interstitial tissue which was quite homogeneous and not cellular.

Indeed, Puricelli admits for some of the changes observed, for instance the cellular increase of the glomeruli, an inflammatory stimulus. His investigations showed further a remarkable absence of casts, only in three cases, and Puricelli admits for these cases also that they might have been complicated by interstitial nephritis. It will therefore be seen that Puricelli's own cases are by no means uniform nor conclusive evidence on the results of venous congestion. On the contrary the certain cases of chronic cyanosis which he described do not agree with his final conclusions. But it is of the utmost importance in a study into the effects of venous congestion upon the kidney to consider various possibilities which may complicate the picture; for this lesion is frequently the result of diseases, like endocarditis with its occasional acute exacerbations and complications, pericarditis, and pleuritis, or like cirrhosis of the liver, which expose the kidney to periodic or terminal infections and inflammatory

irritants. The frequently accompanying arteriosclerosis may further modify the results.

The importance of these factors had already been fully appreciated and discussed by Traube and Bartels, but disregard and lack of proper criticism of subsequent authors reintroduced certain difficulties which older investigators had once successfully overcome. Thus Puricelli's description of cyanotic induration of the kidney which eventually changes to a granular atrophy (Bollinger) became generally recognized in text-books, although differences about many points still continued. These latter concern mainly the nature of connective tissue formation, the cortical changes, and the granular atrophy of the organ. Orth²⁴ described interstitial thickening not only in the medulla, but also in the cortex between tubules and around Bowman's capsule, but questioned whether this represented a genuine increase in fibers, or rather a swelling of fibers with thickening of the interfibrillar cement substance, which would necessarily make the fibrillar structure more distinct and cause an increase in volume. The epithelium of the cortical tubules he described as cloudy and fatty in later stages and possessing distinct cilia. To the latter Lorentz²⁵ had already paid some attention, inasmuch as he held that these lining cilia were a protection for secreting cells to prevent escape of albumen from the cells. During stasis the cells become hydropical, bulge towards the lumen, and partly lose their cilia. The cells devoid of cilia cause albuminuria. Orth mentioned further imbibition of the medullary epithelial cells with blood pigment. He considered cell infiltration around the glomeruli and superficial atrophic areas indirect results of stasis. The latter he believed indistinguishable from the senile atrophy, and he also observed that the first of these changes may be quite lacking even in advanced stasis.

Senator²⁶ also followed mainly the Munich school and recognized essentially three stages of the process: general venous hyperemia with certain consequences already discussed, nutritive results caused by the circulatory impediment, and finally the "Stauungsschrumpfniere" of Bollinger,

which is smaller than normal, pale, has a thickened capsule and some superficial contractions caused by infarctions and localized parenchymatous atrophy induced by the long continued stasis. Schmaus and Horn²⁷ have elaborated these various findings. They have described the interstitial tissue between vessels and tubules as very markedly broadened by a homogeneous fibrillar substance, all vessels — arteries, veins, and capillaries — strongly thickened, particularly the interior of the arteries, and patchy round cell infiltration. Since that time (1893) the knowledge of cyanotic induration of the kidney has practically remained stationary, and the modern book descriptions are only very general and by no means very definite.

Birch Hirschfeld²⁸ discusses the subject very summarily. He mentions the engorgement of vessels and speaks of a general increase in fibrous tissue with partial atrophy (epithelial degeneration in convoluted tubules of subcapsular zone) and shallow retractions on the surface (Stauungsschrumpfnieren). He considers hemorrhages rare, occurring only in intense hyperemia.

Ziegler²⁹ speaks of broadened connective tissue between the tubules, but does not specify its nature and whether it is diffuse or local. He speaks further of occasional slight cellular infiltration, fatty degeneration of some epithelial cells, particularly in the medulla. The glomeruli he considers generally unchanged, only few obliterated and their tubules collapsed.

Kaufmann³⁰ regards the firmness of this kidney due to marked engorgement of the vessels and a simple hyperplasia consisting in thickening and sclerosis of the walls of the veins and capillaries and of the interstitial connective tissue particularly in the medulla, which assumes a glossy appearance. The epithelium of the tubules may undergo more or less fatty degeneration forming the fatty kidney of heart disease. He does not speak of any particular change in the glomeruli, except albuminous transudate into the glomerular capsule; but he does mention the occurrence of hyaline casts and red blood cells in the tubules of the medulla.

Woodhead³¹ speaks of large glomeruli with increased connective tissue nuclei(?), rupture in the tuft and masses of altered blood in the convoluted tubules; wedge-shaped patches of fibro-cellular tissue under the capsule (old infarcts?) extending into cortex enclose some Malpighian corpuscles and tubules. He further describes increase in connective tissue nuclei and fibrillated tissue around the walls of capillaries, and the intertubular vessels with a thickened hyaline wall. Beattie and Dickson³² in discussing the general effects of venous stasis state that it is commonly taught that firmness in chronic venous congestion is due to overgrowth and that this is attributed to the increased lymph supply favoring the growth of connective tissue. Microscopic examination shows that in certain cases, and especially in certain organs, *e.g.*, the lungs, there is an increase of fibrous tissue; but this is by no means common in the other organs in which venous congestion, even in extreme degree, exists. In the lungs they consider it excited by the irritation of blood and carbon particles. They recognize a cyanotic atrophy of organs, in which the majority of cells are destroyed and the organ shrinks irregularly and becomes smaller than natural, but regard it rather as atrophy of disease than atrophy due to pressure. (I am told that Professor Greenfield, head of the Edinburgh School in Pathology, teaches in his lectures that venous cyanosis does not cause a connective tissue hyperplasia, and does not lead to granular atrophy in the kidneys. Unfortunately his views have not gained a wider circulation.) Adami and Nicholls³³ describe the kidney as enlarged and the vessels congested. Bowman's capsule with an albuminous coagulum and the tubules with few hyaline casts. Certain epithelial cells of the descending loops contain blood pigment. In long standing cases the capillary and venous walls appear thickened, there is increase in interstitial connective tissue and occasionally a round-celled infiltration. This constitutes the cyanotic induration. In some instances the secreting epithelium is found fatty. Finally atrophy may result. Similar views are voiced by Aschoff in his recent text-book.

A survey of the findings and opinions of the various investigators which have been presented in outline above shows that this subject still has uncertainties and disputable points and lacks important details in the anatomical changes. It seemed worth while therefore to reinvestigate certain features of the process and to add to previous findings. This appears perhaps even more desirable when it is considered that the problem possesses more than purely local interest, and that the effects and structural changes of venous stasis on a tissue or an organ demand a general theoretical and practical importance.

III. Critic of experimental evidence. — Before proceeding to the consideration of the cases which form the basis of these observations it is necessary to pay some attention to the experimental evidence. It is unfortunate that many experiments which have been performed in this connection have not contributed much of value towards the general knowledge of the effects of renal venous stasis, although they have aided in the elucidation of individual points. Particularly the knowledge of the structural alterations in the kidney has received not only no furtherance, but rather a setback by hasty interpretation and generalization of results. Some of the objections to these experimental conclusions were already voiced long ago by Senator in his monograph on Albuminuria; other objections have developed by recent experiences.

In the first place experiments have been frequently complicated by septic infections, and the results must therefore be largely eliminated; there are others in which asepsis was questionably observed or in which existed other possibilities of infection; and their results must be taken *cum grano salis*. The generally quoted observations of Weissgerber and Perls³⁴ belong to this category. They exposed the kidney and renal vein from behind and produced stasis by tying a rubber band around the vein, thereby narrowing its lumen to one-third the normal. All animals died with septic manifestations in from two to four days, and in ten out of eleven rabbits the kidneys showed few to abundant groups of

micrococci particularly in the pyramids, not in the glomeruli. Micrococci were also found in the thrombotic clots around the ligature of the vein, and it is evident that these micrococci spread from their original focus to the kidney and general circulation.

But disregarding these coarser infections and granting a successful technic, there are several grave differences between experimental and natural cyanosis.

(1.) Experimental stasis is introduced suddenly and severely and usually extends over a few hours or days with normal or even increased aortic pressure (marked collateral circulation). Under pathological conditions, stasis occurs very slowly and gradually with diminished aortic pressure, often increased venous pressure, and extends over a long period (months, even years). In these pathological cases exists therefore the possibility of a much greater adaptation or at least modification of kidney substance and function to changed conditions (Senator).

It follows therefore that the results which have been obtained by a sudden complete obstruction of the renal vein, as exemplified by the observations of Ph. Munk and Buchwald and Litten possess no value as far as the study of chronic cyanosis is concerned. But even those experimental procedures in which only a more or less coarse reduction in the lumen of the renal vein is accomplished, or the experimental heart lesions, are for the reasons stated above by no means exact duplications of the conditions governing pathological cases.

(2.) Any stasis produced by operations on or about the kidney is associated or even preceded by double trauma, partly by the sudden severe circulatory disturbances and partly by the mechanical procedure of the operation. If any one doubts the extreme susceptibility of the kidney to sudden circulatory changes he will only have to examine the urine of athletes, oarsmen, or wrestlers after severe physical strain. Some investigators show changes practically amounting to the findings in severe degenerative exudative nephritis. Such operations have therefore frequently been followed by

firm and extensive adhesions around the kidney, marked thickening of the capsule, and when it is further remembered that an additional injury to the kidney lies in the anesthesia, to which many animals are very susceptible, it becomes intelligible that sometimes severe inflammatory reactions within the kidney have complicated the artificial stasis. Braun,³⁶ for instance, in an experimental study devoted particularly to the structural changes of the cyanotic induration of the kidney published in 1901, exposed in dogs the kidney from behind, ligatured the renal vein to one-third of its normal caliber and killed the animals in from eight to forty-one days. The changes thus produced were out of all proportion to those ever observed by any one in human beings even after prolonged venous cyanosis. After fifteen days in a dog, the operated kidney had already shrunk to one-fourth smaller than the normal (!) and was firmly bound to the posterior abdominal wall. Bowman's capsule was thickened, the neighboring connective tissue much increased, the glomeruli compressed, there existed cloudy swelling of the epithelium in the convoluted tubules with partial desquamation and granular masses in the lumen of these tubules. The next dog developed an abscess, died of pneumonia and can therefore not be used as evidence at all. After twenty-five days, in a third dog, the kidney appeared already completely anemic, the glomeruli were compressed, and the capsule filled with exudate; the connective tissue in the medulla was increased and fibrillar. Again after eight days the kidney was fixed immovably to the posterior abdominal wall; the capsule was thickened, the cortex gray, and the glomeruli were compressed and contained exudate. The connective tissue was quantitatively increased and fibrillar, while the epithelium of the convoluted tubules showed cloudy swelling and fatty change. Similar conditions prevailed in the straight tubules with epithelial desquamation and exudation. After forty-one days the kidney was much diminished in size (!), Bowman's capsule was very much thickened, glomeruli were compressed, and there was marked

proliferation of connective tissue everywhere. The epithelium showed cloudy swelling, fatty degeneration and *coagulation necrosis* (!) and desquamation. These changes were more prominent in the medulla. Braun himself concluded that this is the typical picture of early contracted kidney.

It is quite impossible to regard these cases as illustrations of the anatomical changes in venous cyanosis. Even in points of resemblance they differ in being more general, diffuse, and rapid than ever found in human stasis. Moreover, associated early with degenerative, exudative, and proliferative features in the cortex, much exudation into and early compression of the glomeruli, desquamation, and necrosis of the epithelium, and general anemia and atrophy of the cortex, they present very fundamental qualitative differences. In other words, they are illustrations of true and rapid nephritis. One would at all events hesitate to compare these unusual conditions, the results of these experiments, with those of human cyanosis, for, while they undoubtedly present certain features of it, they show so many points of qualitative and quantitative difference that one could not separate the various components of these pictures and establish their proper relation to each other and to pathological venous stasis.

IV. Anatomical evidence of new material. — The observations of this series were based on fourteen cases of well defined general venous stasis. While these fourteen cases have been elaborated in detail, they do not represent all the studied material, but have been selected from a large number of autopsies in which chronic cyanosis was present. The following tabulation shows their general character. Under the heading of pure cases are included all those in which the effects of remote or recent infections on the kidney could be excluded, while in six other cases various complications existed which offered a possibility of additional structural changes by active infections or other agents. One case only represented a marked sudden congestion with death during

an epileptic convulsion. The ages ranged from thirty-two to seventy-four years; of these one was below forty, four between forty and fifty, four between fifty and sixty, two between sixty and seventy, two over seventy and one unclassified (old man). There were eleven males and three females.

TABULATION OF CASES.

PURE CASES OF LONG STANDING.	RECENT STASIS.	LONG STANDING CASES WITH VARIOUS COMPLICATIONS.
Case No. 2. Old mitral stenosis (healed endocarditis).	Case No. 8. Death during epileptic convulsion.	Case No. 1. Old mitral stenosis with recent vegetations on aortic cusps.
Case No. 3. Advanced brown atrophy of heart.		Case No. 4. Brown atrophy of heart, carcinoma of stomach with liver metastases. Terminal lobar pneumonia with pulmonary abscess.
Case No. 6. Mitral and aortic sclerosis and insufficiency; healed endocarditis.		Case No. 5. Mitral and aortic sclerosis (atheroma). Tuberculosis of lungs, tuberculous ulcers of gut.
Case No. 9. Idiopathic heart hypertrophy.		Case No. 7. Deforming sclerosing endocarditis of aortic valve. Recent vegetation on one of the cusps.
Case No. 11. Deforming mitral sclerosis; healed endocarditis.		Case No. 10. Cirrhosis of liver, terminal tuberculous peritonitis.
Case No. 12. Mitral stenosis (healed endocarditis).		Case No. 14. Mitral and aortic sclerosis (moderate). Carcinoma of esophagus.
Case No. 13. Hypertrophy of heart, advanced fatty metamorphosis and brown atrophy.		

GROSS APPEARANCES OF KIDNEYS.

Case.	Size.	Weight.	Capsule.	Surface.	General Appearance.
1 : 32 years. Male.	Slightly enlarged.	Left, 200 grams. Right, 175 grams.	Not thickened; easily removed.	Smooth, mottled.	Cortex slightly atrophied, mottled in color; vessels at base not thickened.
2 : 70 years. Male.	Left, normal. Right, very small.	Left, 225 grams. Right, 25 grams. (Infarcted.)	Adherent.	Left, with old deep con- tractures (infarcts). Right, irregular; renal artery much thickened.	Left cortex about one-half nor- mal width; stellate veins in- jected; marked arterioscler- osis with infarcts.
3 : 49 years. Female.	Large.	Left, 150 grams. Right, 120 grams.	Not adherent on left; slightly on right.	Smooth and pinkish.	Cortex and medulla in proper relation and markings well accentuated.
4 : 59 years. Male.	Normal.	Left, 130 grams. Right, 130 grams.	Not thickened; easily removed.	Left, smooth, brownish. Right, smooth, deep red with some yellow.	Markings fairly regular; ves- sels injected.
5 : Old man. Age?	Somewhat larger.	Left, 175 grams. Right, 175 grams.	Not adherent.	Smooth, regular.	Cortex and medulla in usual relation; veins prominent, particularly in medulla; few whitish spots in medulla (tu- bercles).

GROSS APPEARANCES OF KIDNEYS. — *Continued.*

Case.	Size.	Weight.	Capsule.	Surface.	General Appearance.
6: 71 years. Male.	Enlarged.	Left, 210 grams. Right, 175 grams.	Not adherent.	Finely granular. (Senile atrophy.)	Atrophy of cortex; poor differentiation between cortex and medulla; veins prominent.
7: 52 years. Male.	Enlarged.	Left, 215 grams. Right, 225 grams.	Not adherent.	Purplish, smooth.	Pyramids markedly injected, otherwise intact.
8: 49 years. Female.	Normal.	Left, 150 grams. Right, 150 grams.	Strips fairly easily.	Slightly irregular.	Cortex slightly atrophied, otherwise appearance of venous congestion.
9: 44 years. Male.	Enlarged.	Left, 210 grams. Right, 225 grams.	Not adherent.	Smooth. Right has a cyst.	Very firm; cortex slightly swollen; pyramids dark blue; markings indistinct.
10: 55 years. Male.	Normal.	Left, 160 grams.	Not adherent.	Irregularly lobulated (fetal) and granular.	Irregularly congested; boundary zone between cortex and medulla marked.

11: 66 years. Male.	Gross description lost.		Not adherent.		Somewhat pitted; dark in color.	Cortex thinner than normal; markings much accentuated; vessels moderately thickened.
12: 40 years. Female.	Normal.	Left, 150 grams. Right, 150 grams.				
13: 60 years. Male.	Enlarged.	Left, 250 grams. Right, 200 grams.	Not adherent.		Smooth, veins injected.	Cortex of good proportion; medullary rays marked, glom- eruli also; vessels moderately thickened.
14: 74 years. Male.	Normal.	Left, 150 grams. Right, 150 grams.	Not adherent.		Smooth, dark reddish; ves- sels much injected; few small cysts.	Cortex narrow; markings dis- tinct; vessels at the base of pyramids thickened.

The gross appearances. — A study of these cases showed that the kidney in typical well established venous congestion is enlarged and firm, has a smooth injected surface, an easily removable non-adherent capsule; and cortex and medulla appear prominent. Some of these factors are variable within certain limits. The enlargement may be almost missing and the weight need not exceed that usually found in approximately normal kidneys, for these factors depend not only upon the stasis but on the amount of edema and stretching of the tissues, and senile and arteriosclerotic atrophies. These same conditions influence weight, color, and differentiation, and markings of cortex and medulla. With much edema they will become irregular and sometimes indistinct. To this must also be added the occasional occurrence of more pronounced fatty changes. A combination of these various factors will give to some cases of advanced stasis a mottled appearance, although the vascular changes will most always outweigh the others; and recognition of the lesion is almost always possible.

Substantiation of the claim that venous cyanosis is followed by granular atrophy of the kidney with thickening of the capsule (*Stauungsschrumpfniere* of the Munich School) has not been found in this series. Moreover, it appears plain from microscopical examination that such an atrophy does not occur as the result of uncomplicated venous congestion. Loss of parenchyma is very limited, in many cases absent, and the tissue is well preserved, although it may show some nutritive disturbances. The accompanying venous engorgement and edematous swelling would at any rate fully compensate the moderate loss of glomeruli and tubules and prevent gross appearance of atrophy and collapse of kidney substance. Whenever the latter make their appearance they seem to be due to the frequently accompanying arteriosclerotic changes, notably the formation of cicatrized infarcts, or senile atrophy. On the contrary, long continued cyanosis rather prevents appreciable loss of kidney substance.

The microscopic findings.

A. Changes in the cortex:

(1.) The Malpighian corpuscles and pericapsular tissue. — The Malpighian corpuscles even in recent stasis are enlarged, show well marked dilatation of the capillary network composing the glomerulus and a distinct lobular arrangement. Associated early is simple swelling of the lining epithelium and endothelium of the capillaries. This simple edematous swelling produced by stasis and occasionally associated with the appearance of fine fat drops must not be identified with the parenchymatous degeneration of inflammatory lesions. In the well established cases the tuft appears always very markedly engorged and fills the greater part of the capsule, often completely. Afferent and efferent vessels are usually very prominent, the afferent vessel occasionally swollen and thickened, and the endothelial cells of the capillaries also swollen. When, however, the engorgement is extreme these cells may appear flattened and thin. In all long standing cases the reflected epithelium over the tuft is markedly atrophic and frequently has been lost. The tightly stretched capillaries, whose lumen and cells are made very definite by their dense blood columns, appear perfectly naked and the tuft is therefore poor in cells. This loss of epithelium, which is very characteristic in long standing stasis, is in all probability the result of a simple pressure atrophy and not associated with any degenerative changes in the wasting epithelium. In the majority of glomeruli the tuft remains free within the dilated capsule, and the latter frequently shows a moderate amount of granular albuminous coagulum, which at times may be considerable. The capsular wall is generally not thickened, but appears simply swollen and hyaline; in many instances, however, it presents remarkable thinning and fibrillar separation. The lining epithelium of the capsule also is either swollen, edematous or elongated and thin, frequently fibrillar. Characteristic is a very marked irregularity in the outline of the capsule. It appears that under the permanent stretching and possible pump action of the much engorged tuft against the capsular

wall the lining epithelium and elastic fibers of the latter become overstretched and atrophic. (Brodie (Renal activity. Harvey Lectures. Lippincott Company, 1910) considers the glomerulus principally a "propulsor." Nussbaum also observed in the newt expansion and contraction of the glomerulus and pulsation with each heart beat.) Thus the lining epithelium is extremely flattened under these conditions. Such thin walled glomeruli show a conspicuous scalloping of the capsule, which in places may assume considerable dimensions. These quantitative changes are at times associated with others, which lead to obliteration of the Malpighian structure. Swelling and serous transudation within the tuft may assume greater dimensions and lead to fusion of groups of endothelial cells and obliteration of capillary loops, with contraction and loss of the glomerulus. Occasionally these swollen endothelial cells fuse with the capsular transudate and swollen lining cells of the capsule to undergo a hyaline transformation which gradually involves the rest of the glomerulus and results in the formation of a small hyaline globule. This change resembles somewhat that seen in nephritis, but remains confined to few glomeruli. Moreover, these changes which lead to annihilation of the Malpighian bodies take place only within original fixed tissue cells. As far as can be seen they are to be explained partly by the vascular pressure and partly by serous transudate and swelling of the cells; and are probably associated with nutritive disturbances in the cells. There is nowhere evidence of inflammatory change; invasion of foreign cells, or the formation of new fixed cells and connective tissue replacement from the capsule. The severer more advanced results described above are not general or diffuse, nor do they appear in all cases even of advanced stasis. Undoubtedly they occur only when the transudation becomes very abundant and is retained in the capsule. In this series they were most conspicuous in Case 1 (exacerbation of fibrous endocarditis) where the possibility of an inflammatory irritant existed. Hemorrhages in the glomerulus by rupture into the tuft are still rarer, although

from dilatation these capillaries may sometimes assume the dimensions of sinuses. There occurs only very rarely complete hemorrhagic destruction of a glomerulus, although not uncommonly there may be blood pigment in the endothelial capillary cells.

The behavior of the pericapsular connective tissue depends somewhat upon the changes within the glomerulus, and the neighboring parts. It is generally edematous and stretched, particularly when the tuft has been lost and the glomerular capsule has collapsed or it may, by a colossal edematous distention, tend to a compression of the glomerular capsule. Lymphatics around the Malpighian corpuscles are occasionally enormously enlarged and engorged with lymph. Sometimes the pericapsular tissue may present a thickened appearance, but this appears solely due to a hyaline fusion of old edematous tissue and not to a new formation of connective tissue. In fact there exists generally absence of any appreciable fibroblastic or lymphoid cell invasion, although in six cases a localized streaky accumulation of lymphoid cells within dilated lymphatics was observed. It occurred in four cases in some of the lymph tributaries of larger vessels particularly the veins: Cases 3, 11, and 13 of pure stasis and in Case 14 complicated by carcinoma of the esophagus. One case (4), carcinoma of the stomach with liver metastases and terminal lobar pneumonia and pulmonary abscess, showed a few localized lymphoid accumulations directly under the capsule, and in one case ((1) endocarditis with acute exacerbation) it was prominent in some lymphatics which accompany the vessels of the loops and collecting tubules from medulla to cortex. In no case were these accumulations diffuse; nor did they assume the character of focal infiltrations, but seemed to depend primarily upon marked lymph stasis. In Case 1 (exacerbation of fibrous endocarditis), where this condition appeared most prominently, the possibility of a superadded infection existed, although no other evidences of such were found. It is certain that many cases of severe stasis occur without any such manifestations.

The changes described above appear to represent the

direct, immediate, and remote effects of chronic stasis in and around the glomeruli. Additional findings which are observed at times and have been described by others are evidently not the results of the circulatory disturbances but of super-added irritative influences. These are parenchymatous degeneration of the glomerular cells and capsular epithelium, an increase in the cell contents within the tuft, general hyaline obliteration of Malpighian corpuscles, and hyperplasia of the pericapsular tissue. In this series cellular increase and plumpness due to cloudy swelling of the tuft was observed only twice: in a case of cirrhosis of the liver with tuberculous peritonitis in which the stasis had evidently been complicated by the effects of an infection, and in a case of mitral and aortic sclerosis complicated by tuberculosis of the lungs and gut. A similar explanation will, I believe, be found applicable in cases where such destruction and proliferative features have appeared during the course of venous hyperemia. In this connection it must also be mentioned that in some cases infarct formation involved isolated Malpighian corpuscles and their immediate surroundings in streaky scar tissue; and that perivascular thickening of vessels at times extended to neighboring pericapsular tissue. Such lesions must, of course, be separated from the changes incident to stasis.

(2.) The convoluted tubules and the intertubular tissue. — The convoluted tubules even in long continued stasis may be remarkably well preserved. Usually they are large, wide, and not distorted. Their basement membrane is definite and not thickened, and sometimes shows hyaline swelling. Occasionally some compression by massive edema may occur. The epithelium of these tubules is generally rather swollen, granular and sometimes sufficiently edematous to bulge distinctly towards the lumen. Desquamation and necrosis never occur in pure stasis. Even the cases complicated by infections left the convoluted tubules relatively clear. In two cases of pure stasis the formation of new nuclei, sometimes leading to multinuclear cells, was plain.

This process was still more evident in the medullary portions of the loops and collecting tubules and will be described further under the heading of medulla. Hemorrhages into these tubules have not been observed.

The capillaries between the tubules show throughout a very marked engorgement which, while generally not equaling the dilatation of the medullary vessels, may in some cases reach equal dimensions. Ectasy of the capillaries is less frequent in the cortex than in the medulla.

The intertubular tissue shows considerable variation in edematous imbibition and stretching. In some cases it may reach great dimensions, but usually it is less and somewhat more irregular than in the medulla. A new formation of connective tissue is absent, and the broadened spaces were seen only in four cases, two of which were complicated by acute infections, to contain a few compressed fibroblasts within edematous tissue.

The larger vessels of the cortex are all markedly dilated and their adventitia generally definitely thickened. This thickening may extend in streaks carrying fibroblasts to some of the adjoining structures. The adventitia shows, further, much edema and the veins particularly are surrounded by dilated lymphatics which occasionally carry lymphoid cells.

Endarterial thickening also occurs, but is less conspicuous and much more variable. In fact it is questionable how much this vascular thickening is due to the stasis alone, as a large number of cases in which cyanotic induration of the kidney occurs are associated with arteriosclerosis and infarct formation. The cases of this series showed considerable variation which did not seem to correspond directly to the degree or length of the cyanosis.

B. The changes in the medulla :

(1.) The vessels and interstitial tissue. — The same conditions prevail essentially in the medulla that exist in the cortex, only with more accentuation and some modification. The medullary vessels as well as all capillaries and the

lymphatics show the effects of venous cyanosis in colossal and early dilatation, which in the capillaries frequently amounts to a true ectasy. This much greater involvement of the medulla becomes intelligible on anatomical grounds, for part of the branches of the renal artery enter directly into the capillary system of the medulla without passing through the glomeruli at all. This causes necessarily a much greater venous tension in these structures, while the cortex, and particularly the glomeruli, are least under venous pressure. On the other hand, they suffer more from decline in aortic pressure.

In advanced cases where the edema of the interstitial tissue has become very massive, compression of certain vascular districts may take place, the injection of the vessels appears then more irregular. The dilated lymphatics surrounding the loops and collecting tubules are prominently dilated and in one case were filled with lymphocytes.

The character of the interstitial tissue can be particularly well studied in the medulla. It is markedly broadened and presents a very characteristic structure. It is made up of a very delicate felt-like network of fine fibrils, which appear to be derived by a gradual edematous stretching from the original connective tissue and seems to carry much cement substance within its meshes. At times it assumes an almost homogeneous hyaline appearance, due to fusion and possible coagulation of its constituents. Evidences of proliferation or hyperplasia of this tissue can nowhere be made out, and in fact there exists a remarkable absence of any nuclei. This increase of interstitial tissue, which has already been well described by Schmaus and Horn, occurs therefore entirely at the expense of old connective tissue and constitutes no hypertrophy or hyperplasia in the usually employed sense. It must be attributed solely to the results of edematous swelling of the parts.

(2.) The loops and collecting tubules. The formation of hyaline casts. Epithelial proliferation.—The tubules of the medulla for reasons explained above are apt to suffer

somewhat more from the severer effects of stasis than those of the cortex. Still even in pronounced cases they never show those destructive or degenerative changes which occur in the inflammatory conditions. They may be at times compressed, particularly the thin loops, and in very rare instances, in isolated tubules, their swollen epithelium may fuse to hyaline casts. More often these cells show moderate edematous swelling and some vacuolization, but on the whole, except when they become connected with casts, they retain their individual outline. It is common to find granular blood pigment within the lining cells, particularly in the collecting tubules; and at times irregular clumps of blood pigment and shadows of red blood cells resulting from hemorrhage may be seen to fuse into homogeneous casts within these tubules. It is evident that these gradually lose their hemoglobin contents as they may be found in all shades to the character of hyaline casts. However, the largest number of casts, which usually appear abundantly in the loops and particularly the collecting tubules, owe their origin to other sources, as they occur in good numbers even when hemorrhages or hyaline fusion of compressed tubular cells cannot be demonstrated. They are missing in recent stasis. These genuine hyaline casts are never found in the upper part of the tubules; and this, along with the fact that the amount of transudation into the capsules seems to stand in no relation at all to the number of these casts, speaks decidedly against their derivation from coagulated and fused albuminous transudate from the glomerular capsule. It is well known that their origin has been the cause of much controversy, and that even to-day there exists no uniformity of opinion about their source.

Originally it was claimed by earlier investigators, Ph. Munk³⁶ and Burkart,³⁷ that the occurrence of hyaline casts was an indication of inflammatory action and never occurred in pure stasis. These views were later contradicted by Erythropel,³⁸ who claimed to have found them seven hours after tying of the renal vein. But Weissgerber and Perls properly remarked that none of these observations were

applicable to the solution of the problem under discussion because the conditions in the kidney resulting from ligation of the vein are totally different from the results of pathological venous congestion. To that must be added that hyaline casts are not composed of ordinary fibrin. Later experiments have demonstrated hyaline casts in the urine after either short ligation of the vein (one-half hour) (Vorhoeve,³⁹ Ribbert⁴⁰) or after simple reduction in the caliber of the renal vein (Posner,⁴¹ Litten⁴²). These experiments were, however, most always followed by severer changes in the renal epithelium and the lumen of the tubules was filled early by granular cellular detritus, which later might fuse to casts (Litten). They resemble, therefore, more closely the conditions which exist in nephritis, and it cannot be supposed that this process can be the main source of these casts in renal stasis. Posner's experiments showed milder lesions. After a few hours of partial reduction of the vein the glomeruli and tubules contained only granular albuminous precipitate, while some columns of the epithelial cells were well preserved. After twenty-four hours all the tubules contained homogeneous hyaline casts. Posner believed that casts owe their origin largely to transudation, but he did not eliminate entirely red or white cells as contributory elements. He denied their origin from epithelial cells, or from a croupous fusion of epithelial cells with lymph or exudate.

Ribbert sought to prove the derivation of these casts by the injection of a carmine solution into the jugular vein of a rabbit thirty-five minutes after removal of a ligation of the renal vein which had been in operation for one and a half hours. Autopsy showed Bowman's capsule and parts of the tubules filled by coagulated reddish albumen, and a few almost colorless hyaline casts, though the epithelium of some tubules was filled with carmine particles. Ribbert concludes that the almost colorless casts (not entirely so, for they are described as with a reddish tinge) and albumen could not be derived from the epithelial cells. This is, however, not a convincing proof, for it is quite possible that disintegration of epithelium might produce certain changes in the carmine

stain; especially as the casts and albumen masses were slightly reddened.

Of great value for the understanding of the formation of these casts has been the direct anatomical observation of diseased organs. It is now well settled by these observations that hyaline casts are not characteristic of inflammatory changes only. They occur with great regularity and sometimes in abundance in non-inflammatory diseases, particularly in advanced chronic cyanosis, and to a lesser extent in the amyloid kidney; they are common in icterus, cholera, and even long continued constipation and arteriosclerosis. As regards their origin Senator in particular strongly denied their derivation from serum albumen, at least pure serum albumen, and traced them to desquamated and fused epithelial cells, or at least albuminous lymph which has been mixed with degenerating epithelial cells (Weigert⁴³). Similar views had already been expressed by Ottomar Bayer in 1868,⁴⁴ who claimed to have observed the transformation of epithelial cells through all stages to hyaline casts, while Rindfleisch, Klebs, and Bartels regarded them mainly as results of exudative changes, but admitted that red blood cells might enter into the formation of some, particularly the colloid (Klebs) and waxy (Bartels) casts.

Senator based his objections to the derivation of hyaline casts from exuded blood serum upon several factors: first, their chemical composition, which shows them not to be fibrin. Second, the lack of parallelism between albuminuria and cast formation, and the fact that in chyluria the urine is particularly poor in casts. Third, the antagonistic property of urine to coagulation of serum. Fourth, the scarcity of casts in amyloid disease. Senator doubtless went too far in an endeavor to trace all hyaline casts to epithelial fusion and in the absolute importance of his objections to other sources. His conclusions have been critically reviewed in this respect by Weissgerber and Perls.

Observations of Axel Key,⁴⁵ Litten, Weissgerber and Perls, Cornil,⁴⁶ Lépine, Orth, and others seem to have demonstrated the occurrence of a physically similar substance between the

membrana propria of the epithelial cells of the tubules, and further that hyaline or colloid globules may appear within cells to be discharged and fuse to hyaline or colloid masses. These have been termed secretion casts. It is questionable whether some of these changes were not artefacts or degenerative cellular changes.⁴⁷

The observations of the cases comprising this series have demonstrated that a large number of hyaline casts in stasis owe their origin to transudation of stagnant lymph through and between swollen edematous epithelial cells in the lower parts of the loops and collecting tubules, and that occasionally such cells are torn from their basement membrane and consumed with the hyaline masses. This process was observed in various stages. At first the coagulated masses were still attached to the surface and between the epithelial cells. Recently torn off casts presented, therefore, a peculiar punched out periphery, were indented and stellate, corresponding to the shape of the lining cells between and through which they passed. Occasionally these recent casts were seen to contain broken off fading epithelial cells. This was particularly well shown when these cells had originally contained blood pigment, which was also consumed in the hyaline mass. On the downward road these casts were gradually compressed to rounded masses and their edges smoothed off.

The coagulation of this transuded albuminous lymph seems to be accomplished by its contact with the edematous swollen epithelial cells of the tubules; and the idea of a ferment action seems very probable, for these cells themselves are considerably altered by autolytic processes, or by other changes due to long-continued edematous imbibition. Lymph is easily coagulable and it is also likely that stagnation further increases the coagulability of the lymph. While it seems certain, therefore, that the bulk of the hyaline casts originate in venous congestion as just described, the evidence also shows that some may either become mixed with epithelial remnants, blood cells or blood pigment, and that casts may owe even their entire origin either to fused hemorrhages or desquamated compressed epithelium from

the loops. The latter formation, however, must be regarded as rare in stasis and only incidental.

There remains the matter of epithelial proliferation, which was particularly marked in two cases of this series, and has already been referred to in connection with the convoluted tubules. As far as I can see this change has not been reported in the observations on chronic cyanosis of the kidney. We know by recent investigations that proliferation of epithelium in nephritis and degenerative lesions of the kidney is a rather constant phenomenon and may reach considerable dimensions amounting to a diffuse infiltration of the tubules (particularly loops and collecting tubules) by newly formed epithelial masses. In the convoluted tubules there occurs more often the formation of a new syncytial lining or the formation of giant cells. This subject has been reviewed by me⁴⁸ and recently by Wittich,⁴⁹ where discussions of the matter may be found. These changes in stasis are not so extensive or diffuse, they are confined to a more or less abundant formation of new nuclei with the formation of multinuclear cells; they are restricted to the cells lining the tubules and occur mostly by amitosis. The formation of typical multinuclear giant cells, frequent in nephritis, is missing, but occasionally a syncytial lining is found. These findings and points of difference between the epithelial proliferation in nephritis and chronic cyanosis seem to indicate that this process in stasis is evidently of a reparative nature in contradistinction to the nephritic proliferation which has all the peculiarities of a diffuse catarrhal inflammatory parenchymatous proliferation.

V. Albuminuria. — The albuminuria in venous cyanosis of the kidney demands special consideration in view of the anatomical findings recorded above. It is well known that there has been considerable discussion regarding the source of serum albumen and the exact mechanism by which it enters the urine.

Two possible sources have been mentioned. Based particularly on experimental evidence, Senator⁵⁰ held that

albumen transuded directly from the interstitial capillary and lymph system into the tubules. In this connection he with Lecorché and Cornil attributed considerable importance to damaged epithelial cells. Now while this may occur experimentally in very rapidly produced, severe venous compression with strong or at least normal aortic pressure, where cells and basement membrane are likely to be much injured, certain objections, of which I have already mentioned some, argue strongly against any appreciable transudate of fluid albumen directly from the capillary or lymph system into the tubules. Anatomical observations of pathological cases have demonstrated that lymph (not pure blood serum) passes slowly and very gradually from the interstitial lymphatics through the swollen, hyaline membrana propria of the tubules. It passes between and through the swollen lining epithelial cells into the lumen, where it coagulates to casts.

But much evidence supports the view that at least the largest amount of the fluid serum albumen transudes with the urine water through the glomeruli. We find in the dilated glomerular capsule an abundant coagulated transudate. It is, however, not definitely settled by what mechanism this occurs.

The excellent experiments of Runeberg⁵¹ demonstrated conclusively that the older view, which attributed the albuminuria of stasis to increased pressure, was erroneous, but on the contrary, albumen filtered better under low than high pressure. Posner added to this the necessity of slowing of the blood current. Both of these factors enter into the condition within the glomeruli during pathological venous stasis; since the glomeruli, for reasons previously explained, are least affected by the increased venous pressure (some authors have even denied any influence), while they necessarily suffer most from the decline in aortic pressure and slowing of the blood current. However, it has been doubted for various reasons whether these more or less purely mechanical conditions are sufficient to explain the increased transudation of serum albumen through the capillary loops of the glomerulus. Many modern writers have therefore returned

to the ideas of Heidenhain,⁵² Cohnheim,⁵³ and others, that albuminuria represents primarily a lesion of the lining epithelium of the tuft. Krehl,⁵⁴ for instance, holds that opinion. The anatomical evidence here recorded does not lend any support to this conception. In practically all advanced cases of stasis the epithelial lining of the glomeruli is either entirely lost or markedly compressed and atrophied. Still the albuminuria in these instances does not seem to increase to very large amounts as one would expect, but always remains quite moderate and its variations appear to depend very directly upon the circulatory conditions. This direct influence is so apparent that one can hardly assume a correspondingly rapid regeneration of this epithelium. Furthermore, microscopic examination does not reveal a greater abundance of albuminous transudate as the epithelium wastes, even in those glomeruli which have completely lost their lining epithelium and appear as naked capillaries. In some the capsule may not be dilated nor filled with albuminous transudate, showing that the transuded amount must have been small and directly discharged as under normal conditions.

But this loss of epithelium has still another interest. It does not appear that glomeruli devoid of their epithelium have lost their functioning capacity, but simply transude a somewhat more albuminous fluid than under normal conditions, a state of affairs which argues against any specific secretory activity of the glomerular epithelium. The anatomical evidence here presented therefore lends weight to the belief that the appearance of serum albumen in the urine during the course of chronic venous congestion depends mainly upon the circulatory disturbances in the glomeruli which become associated with certain nutritive changes in the endothelial cells and in the intercellular substance. These changes lead to increased permeability of the capillaries to serum albumen. The epithelium of the tuft seems not to be concerned in this process.

VI. SUMMARY.

1. The kidney of chronic venous stasis is primarily a moderately enlarged organ, firm and congested with definite markings and usual relations of cortex and medulla.

2. In advanced cases edematous imbibition may lead to marked enlargement and increase in weight of the organ, and to irregular evidences of congestion. Occasionally it may produce more or less obliteration of markings which are replaced by a pale moist appearance, swelling of the parts and, if nutritive changes become prominent, by yellowish (fatty) spots. It may under such conditions be confounded with nephritis.

3. Microscopical characteristics are: engorgement and enlargement of glomeruli; loss of glomerular epithelium; albuminous transudate into the capsule, which, if retained, may in rare instances fuse with the edematous swollen cells of tuft and capsule, and the whole undergo subsequent hyaline metamorphosis; hyaline swelling or thinning and scalloping of capsule; edematous stretching and fibrillation of the intertubular tissue most prominent in the medulla; dilatation of capillaries to ectasy, also most prominent in the medulla; dilatation of the lymphatics particularly around the veins, loops, and collecting tubules, which become filled with stagnant lymph and occasionally with lymphocytes; edematous imbibition and moderate granular and fatty metamorphosis of tubular epithelium; formation of new epithelial nuclei leading to the formation of multinuclear cells and syncytial lining in the tubules, particularly in the loops and collecting tubules; occasional hemorrhages in the medullary tubules; blood pigment in the epithelium of loops and collecting tubules; hyaline casts mostly derived from transuded lymph with some from desquamated epithelium and from hemorrhages in loops and collecting tubules.

4. (a) Focal or diffuse inflammatory cellular foci do not occur; (b) a new formation of connective tissue does not occur; (c) granular atrophy of the kidney does not occur.

5. Larger blood vessels show usually adventitial thickening and edema, also rather irregular endarterial thickening. These are variable findings in pure cases.

6. Chronic cyanosis occurs frequently in connection with arteriosclerosis, productive nephritis and senile atrophy. Such combinations present complicated anatomical pictures. Acute or subacute infections may also introduce additional structural changes to an existing stasis.

7. The accompanying albuminuria depends upon an increased transudation of albuminous fluid through the glomeruli.

8. It does not appear that the lining epithelium of the glomerular tuft has any connection with the albuminuria, or that it exercises any influence over the transudation from the glomerulus.

9. The term "cyanotic induration" should not be employed in the ordinary sense of connective tissue hyperplasia; if used at all it should only allude to the physical character of the kidney.

10. These investigations uphold in general the original contentions of Traube uttered in 1856.

VII. ADDENDA: PROTOCOLS (ABBREVIATED).

Case No. 1.—No. 1907. J. C., male, age 32, 169 centimeters, 128 pounds. Died Dec. 16, 1910; autopsied Dec. 16, 1910. Old standing mitral stenosis (mitral admits only tip of finger). Mitral segments adherent to each other, the upper margin of the cusps covered by numerous vegetations. The segments of the aortic valve with more recent fibrinous deposits. The tricuspid valve markedly dilated admits five fingers. Heart, weight, 700 grams. General venous stasis. Ascites.

Left kidney, 200 grams; slightly enlarged. The capsule strips easily and is not thickened. Cortex slightly atrophied, mottled in color, some parts red, others pale. The vessels at the base not thickened. Right kidney, 175 grams; similar to the left.

Microscopic examination. Cortex: The Malpighian corpuscles are generally large. The capillary tufts engorged with blood fill the greater part of the capsules. The efferent and afferent vessels of the tuft are very prominent, the endothelial cells of its capillaries are swollen while the reflected epithelium over the tuft is generally lost (pressure atrophy); the tuft appears therefore poor in cells. The epithelium lining the capsule, however, is frequently swollen and edematous, but in other places elongated

and fibrillar. In the majority of glomeruli the tuft remains free within the capsule. The capsular wall is generally not thickened, but rather thin and hyaline. Some glomeruli show an attachment of a capillary lobule or lobules to a portion of the wall, either by fusion of tuft cells with those of the capsule, by transudate, or possibly both. These attached parts fuse to hyaline masses and may thus gradually involve a considerable portion of the glomerular tuft. In some other Malpighian corpuscles one observes much edematous swelling of the capsular epithelium, which in these instances appears to be lifted off its basement membrane by transuded fluid. In relatively few instances tuft and capsule have been completely transformed into a small, collapsed hyaline globule, but the connective tissue invasion from the capsule, frequently seen in nephritis, is entirely absent. Occasionally atrophy of the tuft seems to follow in some glomeruli fusion of swollen endothelial cells, obliterating capillary loops. Around collapsed Malpighian corpuscles the tissue appears much stretched, edematous, and swollen. A general pericapsular cellular infiltration is absent, but a resemblance to such a lesion is produced by a localized streaky marked dilatation of interstitial lymphatics with lymph stasis and containing many lymphoid cells which surround the loops and collecting tubules. These lymphatics extend upwards from the medulla, accompanying their vessels, and having reached the cortex are seen in the neighborhood of some glomeruli, giving at first sight the impression of inflammatory foci. That this, however, is not the case is shown by the fact that the convoluted tubules in the immediate neighborhood as well as generally are quite intact and free from such foci; their intertubular spaces, although markedly engorged and somewhat edematous, show only few compressed fibroblasts. Nowhere can be seen any new formation of connective tissue. The epithelium of these tubules shows only slight granular degeneration. The larger vessels between cortex and medulla are moderately thickened; in some the intima considerably.

Medulla: Vessels very markedly dilated, the interstitial tissue edematous, swollen, but not cellular. The epithelium of the tubules intact. In places it is rather turbid, and frequently the lining cells contain granular blood pigment. Irregular clumps of granular blood pigment gradually fusing to homogeneous casts are seen particularly in the collecting tubules. These appear to be due to old hemorrhages. Other casts are plain and hyaline and are evidently derived from transuded serum discharged from the lymph stream between and through edematous swollen epithelial cells. Recent casts present, therefore, frequently a very irregular, punched-out periphery, where the cast mass passed through or between cells, or where they have broken off from epithelial cells to which they were originally attached; other evidently older ones are rounded off. Occasionally casts are thus seen to contain epithelial cells which have been carried off and are gradually fused with the transuded masses. Some of the loops are much compressed by interstitial edema and their cells seem to fuse into hyaline masses by desquamation. But this process appears very limited.

The interstitial tissue throughout the medulla shows the edematous swelling described above but without fibroblastic increase, and the dilated lymphatics with stagnant lymph and lymphoid cells appear here as in the cortex around the loops and collecting tubules.

Case No. 2. — No. 1904. R. J., male, age 70, 175 centimeters, 158 pounds. Died Dec. 14, 1910; autopsied Dec. 14, 1910. Old productive obliterating mitral endocarditis (mitral stenosis). Mitral admits only tip of finger. Right ventricle somewhat hypertrophied. Left auricle almost completely filled with ante-mortem laminated thrombus taking the shape of the left auricle. The right side of the heart much distended by post-mortem clots. Left ventricle, 15 millimeters in thickness, muscle pale red. The aortic cusps moderately thickened and their ventricular surface just below the line of contact with many apparently fairly recent vegetations. Weight, 1,000 grams.

Left kidney, 225 grams. Surface with old deep contractures. On section firm, slightly pale, cortex about one-half normal width. Stellate veins injected. Veins tortuous. Vessels at the base prominent. Right kidney extremely small, about twice the size of an English walnut. Weight, 25 grams. Renal artery much thickened and narrow but patent. On section this kidney contains almost no parenchyma. Its capsule strips with difficulty, apparently the result of old very complete infarction.

Microscopic examination of left kidney: The glomeruli appear irregular in size and shape although mostly large and engorged as in Case No. 1. The edematous swelling of the endothelium of the tuft is marked in some glomeruli, leading to fusion of cells, obliterating the lumen of the capillaries and is followed by edematous swelling of the capsule and adhesion of the tuft to the swollen epithelium of the capsule with hyaline obliteration of the glomerulus as in Case No. 1. The capsular space shows frequently moderate escape of serum into the capsule. The capsular outline appears irregular, slightly scalloped. The vessels are much dilated, the larger ones thick-walled, edematous. There are few streaks of scar tissue as the result of obliterated vessels and old infarcts, carrying few small hyaline glomeruli. The interlobular tissue in the cortex is swollen and edematous, but shows no cellular or fibrous increase. The convoluted tubules appear large and show some granular degeneration. In the medulla the vessels are very markedly engorged, the interstitial tissue very broad and edematous. The interstitial medullary tissue assumes therefore a very characteristic appearance. It is made up of a very delicate felt-like network of faint fibrils, which are probably derived by gradual edematous stretching from the original connective tissue and carries fluid within its meshes. At times it has an almost homogeneous hyaline appearance, probably due to coagulation of its liquid constituents. This connective tissue has no nuclei and nowhere is any evidence of new connective tissue formation. Lymphatics around tubules are frequently enormously dilated and filled with homogeneous coagulum. Groups of tubules are compressed by

massive interstitial edema, and the engorgement of vessels in such parts appears more or less circumscribed, the result of pressure from neighboring parts (this accounts for the pale, moist appearance of portions in the gross appearance). The epithelium of the medullary tubules appears well preserved, but in parts shows edematous hyaline swelling and often contains blood pigment. Collecting tubules and the loops contain many hyaline casts. Their most important source seems to be serum. This may be seen to pass through the tubular wall between and through edematous swollen cells. The coagulated transuded serum at first remains attached to the protoplasm of the cells through which it passed. In breaking off the cast frequently assumes a very distinctly stellate indented appearance, the punched-out portions of its periphery corresponding to the form of the lining cells. On the downward road the edges gradually become smooth and rounded off. Many are seen to tear lining cells with them and transform them, and frequently additional red blood cells or blood pigment contained in the torn-off epithelial cells, into a hyaline mass.

Case No. 3. — No. 1902. A. S., female, age 49, 167 centimeters, 75 pounds. Died Dec. 8, 1910; autopsied Dec. 8, 1910. Atrophy of brain with productive meningoencephalitis. Advanced brown atrophy. Fat infiltration of the heart and collapse of heart. All auricles and ventricles filled with fluid blood. Tricuspid orifice admits three fingers and mitral two. Weight, 200 grams. Lungs show advanced atrophic emphysema. General visceral stasis.

Left kidney, 150 grams, large. Capsule peels easily. Surface smooth and pinkish. Stellate veins distinct. On section cortex and medulla appear normal in size and relation and the markings well accentuated. Right kidney, 120 grams. Capsule on this side slightly more adherent, otherwise like the left.

Microscopically: Glomeruli large and engorged. The lobes of the tuft very distinct. Epithelium lost. Tuft fills the capsule, which shows very definite scalloping, hyaline thickening and fibrillation giving it a characteristic coast-line appearance. When the elastic fibers appear thus overstretched the glomerular capsule may show hernia-like indentations. There also exists hyaline swelling and fusion of the endothelium in parts. Very occasionally occurs rupture of capillaries within the tuft. The lymphatics around some larger veins are dilated and filled with lymphoid cells in streaky distribution (lymph stasis). However, there is no general infiltration with such or other cells around glomeruli or tubules. There exists much edema in the cortex with interstitial stretching leading to unusually marked separation of the tubules, which are otherwise well preserved. Blood vessels between the tubules are generally much engorged and the tubular cells swollen and edematous. The convoluted tubules and particularly loops and collecting tubules show the formation of new nuclei leading by amitosis to multinuclear cells, or sometimes a syncytial lining of tubules. These epithelial cells contain

blood pigment. The lumen of these tubules shows hyaline casts. The interstitial tissue in the medulla shows extreme edematous stretching as previously described. The larger vessels appear moderately thickened, but no endarterial proliferation.

Case No. 4. — No. 1957. E. J. F., male, age 59, 173 centimeters, 88 pounds. Died March 14, 1911; autopsied March 17, 1911. Carcinoma of stomach with extensive metastases in liver. Terminal lobar pneumonia with pulmonary abscess. Brown atrophy of heart, 225 grams. Small, soft, flabby. Tricuspid admits four fingers. Mitral two. Musculature brown, very friable. Valves intact. General visceral stasis.

Left kidney, 130 grams. Firm. Differentiation of cortex and medulla rather indistinct. Capsule not thickened. Strips easily and exposes smooth, pale brown surface. The cortical markings fairly regular. The right kidney, 130 grams, shows marked injection of vessels giving deep red appearance to the kidney. Some areas rather pale yellow.

Microscopically: Large, irregular, scalloped Malpighian corpuscles with thin capsule and a characteristic coast-line like outline. The glomeruli generally engorged usually fill the capsule almost completely. There appears frequently hyaline swelling of capsular epithelium but no fibrous thickening. In some areas around much engorged vessels there is marked streaky lymph stasis with accumulation of lymphoid cells. Some completely hyaline glomeruli within fibrous streaks (infarcts); few with hyaline fusion of tuft and capsule and surrounded by edematous tissue; some areas of fibrous edematous thickening around arteries which sometimes extends to neighboring glomeruli, but generally there exists no thickening around the glomerular capsule. Lymph stasis, edema, and streaky lymphoid cell infiltration appears particularly prominent directly under the superficial capsule.

Case No. 5. — No. 1935. S. W., old man, age not given. Died Feb. 3, 1911; autopsied Feb. 4, 1911. Heart, 360 grams. Generally dilated. Tricuspid admits four fingers. Mitral thickened with calcareous deposits. The aortic valve ditto. The anterior cusp with prominent calcareous projection. The muscle tissue brownish red, friable, hypertrophied. There exist also small caseating tubercles through the lungs and tuberculous ulcers of the large gut. Grossly liver and kidneys show passive congestion. Right kidney, 175 grams. Surface regular and smooth. Capsule strips easily. On section cortex and medulla in usual relation. Stellate veins prominent. The vessels of the pyramids injected. Markings somewhat obscured. Here and there few millet-sized whitish spots at the tip of medulla. Left kidney, 175 grams, same as the right. The capillaries appear much engorged in the medulla, its interstitial tissue very edematous, stretched and fibrillated, the tubules large, its epithelium intact and in the collecting tubules in places vacuolated. Occasional distinct hemorrhage and fusion with formation of casts in the collecting

tubules. The glomeruli injected, some rather turbid, more or less irregular in size and outline, but on the whole well preserved. Few are hyaline. There exists moderate adventitial thickening and edema of the vessels, very localized thickening around them and a few recent tubercles in the cortex. The intertubular tissue in the cortex only moderately edematous, but the tubular epithelium rather swollen and granular.

Case No. 6. — No. 1844. C. R., male, age 71, 82 centimeters, 180 pounds. Died July 25, 1910; autopsied July 25, 1910. Marked aortic and mitral sclerosis. Insufficiency of both valves. All chambers of the heart markedly dilated especially on right side. Tricuspid admits five fingers. Mitral three fingers. Aortic cusp with extensive calcareous sclerosis. Weight, 550 grams. Marked general atheroma of aorta and coronary arteries. Left hydrothorax. Obliterated right pleural cavity. Emphysema. Left kidney, 210 grams. Firm. On section shows atrophy of cortex. Poor differentiation between cortex and medulla. Veins prominent. Capsule strips easily; surface finely granular. Right kidney, 175 grams, ditto.

Microscopically: The capsule on the surface of the kidney thin. Glomeruli large, injected. The capsular space filled with granular albuminous precipitate. The endothelial cells of the engorged tuft swollen. The capsule in parts thin and atrophic or slightly hyaline thickened, in places much scalloped and the whole Malpighian body extremely irregular in outline. The tubules large, their walls thin and distinct and the interlobular capillaries much engorged. The epithelium frequently swollen and granular; there is no cellular hyperplasia anywhere. There is considerable thickening and engorgement of the larger vessels. In the medulla edema and stretching of the interstitial tissue. No cellular proliferation but marked engorgement of capillaries. The collecting tubules contain casts. The capillary engorgement is irregular, interchanging with pale edematous portions possibly due to pressure of one upon the other.

Case No. 7. — No. 1811. E. S., male, age 52, 176 centimeters. Died May 12, 1910; autopsied May 12, 1910. Deforming sclerosing endocarditis of the aortic valve. Recent vegetation of one of the cusps. Hypertrophy and dilatation of left ventricle. The tricuspid orifice admits four fingers, the mitral admits three fingers, the right ventricle measures five millimeters, the left 2 centimeters. The musculature firm, brownish red. Weight, 800 grams. Endarteritis obliterans of posterior coronary artery. Double hydrothorax. General visceral stasis. Left kidney, 215 grams. Dark purplish red. The pyramids markedly injected. The capsule strips easy. The right kidney, 225 grams, like the left. The glomeruli large and engorged. Outline of Bowman's capsule very scalloped, zigzagged, coast-line like. The capsule itself not thickened, but shows a hyaline swelling. The glomerular tuft fills the capsule for the most part. Its lobes are distinct. There is no evidence of a lining epithelium (atrophied and lost), but the endothelium of the tuft is swollen. There is nowhere

to be noticed any increase in cells of the tuft; the capsule contains an albuminous transudate. Occasionally there exists atrophy of the tuft (from pressure). No thickening of or around the capsule and no hyaline transformation of Malpighian corpuscles. No cellular infiltration anywhere. The capsule on the surface of the kidney thin, stretched, fibrillar. The tubules are wide, the cells edematous but otherwise well preserved, the tubular walls not thickened but rather with a hyaline edematous swelling. There occurs in places considerable stretching of the edematous interstitial tissue as previously described. The interlobular capillaries are much engorged, as also the larger blood vessels the outer coats of which are only moderately thickened. Relatively few hyaline casts in loops and collecting tubules.

Case No. 8. — S. C., female, age 49. Died Aug. 28, 1910; autopsied Aug. 28, 1910. Death during epileptic convulsion. Cardiac atrophy. Cerebral edema. Hypostatic pneumonia. General venous stasis. Otherwise well nourished woman. No other reason found to account for sudden death. Heart, 350 grams; small. Tricuspid admits three fingers. Mitral two fingers. Coronary arteries intact. The left kidney, 150 grams; slightly reduced in size. Capsule strips fairly easily. Surface slightly irregular. Cortex slightly atrophied. Appearance of venous congestion. Right kidney, 150 grams, ditto. The glomeruli large and intact. There exists moderate dilatation of the capillaries with swelling of epithelium and endothelium of the tuft. Considerable engorgement of the medullary capillary vessels, but less and irregular in the cortex. The larger vessels are engorged but not particularly thickened. The tubules appear intact and there are no casts.

Case No. 9. — No. 1826. F. K., male, 44 years, 160 centimeters, 141 pounds. Died June 12, 1910; autopsied June 15, 1910. Idiopathic cardiac hypertrophy and dilatation. Heart very large, 600 grams. Tricuspid admits four fingers easily. Right ventricle measures 3 millimeters. Mitral admits three fingers easily. Left ventricle 1 centimeter in thickness. The aortic valve only slightly thickened. Heart muscle friable, brownish, marked fatty infiltration of capillary muscles (tigering). Necrotic cyanosis of liver with jaundice. Hydrothorax. General stasis.

Left kidney, 210 grams. Capsule strips easily. The organ is very firm. On section cortex slightly swollen. Pyramids dark blue. Markings indistinct. Right kidney, 225 grams, like the left, and with a cyst.

Microscopically: The Malpighian corpuscles large, the tuft large, much engorged, but for the most part free in the capsule, which frequently contains albuminous transudate. The endothelial cells of the tuft appear flattened, thin, compressed by blood, and turbid. There appears to be no epithelium left. The capsule shows elastic hyaline thickening, as a rule is well shaped and has no fibrous or cellular thickening around it. There appears atrophy and loss of the tuft in places probably due to

pressure atrophy, but no hyaline change or attachment of tuft to the capsule. The vessels between the convoluted tubules are engorged. The intertubular tissues, however, not thickened, practically unchanged, the tubules large, cells swollen and edematous but otherwise well preserved. There is marked but irregular engorgement of the vessels in the medulla. The cells of the large collecting tubules contain definite blood pigment. There exists distinct but not excessive edema of the intertubular tissue. The cells of the tubules are well preserved and the larger arteries and veins moderately thickened.

Case No. 10.—No. 1667. P. K., male, 55 years, 167 centimeters. Died Jan. 12, 1910; autopsied Jan. 14, 1910. Atrophic liver cirrhosis, ascites, tuberculosis of peritoneum. Heart, 400 grams. Marked dilatation of the right side. The tricuspid admits four fingers, the mitral two fingers. Left ventricle 14 centimeters in thickness. Heart muscle brownish red. Left kidney, 160 grams. Capsule strips easily, the surface irregularly lobulated (fetal lobulation) and granular. The boundary zone between cortex and medulla marked. Irregular congestion. Right kidney, 160 grams, like the left.

Microscopically: The Malpighian corpuscles large, the tuft also. Engorged, turbid, cellular. Some capsular walls moderately thickened and swollen, often wavy and irregular in outline. Vessels between the tubules engorged. The intertubular tissue between well-preserved tubules moderately thickened with fibroblasts. The collecting tubules contain in places hyaline blood casts (old hemorrhages). There exist few hyaline glomeruli and occasional attachment of a glomerulus to the capsule with connective tissue thickening of that part of the capsule. There are hyaline casts in the collecting tubules and loops. Moderate thickening of larger vessels.

Case No. 11.—No. 1700. E. H., male, 66 years, 172 centimeters, 139 pounds. Died Feb. 7, 1910; autopsied Feb. 9, 1910. Deforming sclerosis of mitral valve, hypertrophy of the heart and dilatation of its right side. Cyanotic induration of liver, spleen, and kidneys. Double hydrothorax. Healed calcareous tubercles at both apices with pleural adhesion. General edema. Kidneys microscopically: The glomeruli rather irregular in size, though many large and fill the capsule, some engorged, others less so, and the lobules of the tuft well separated, not cellular, the capsule irregular in outline. In places the tuft appears rather turbid and its cells swollen. The capsular space contains granular coagulum, and the walls show either a hyaline swelling or a thinning of the elastic fibrils, which may become so extreme as to amount to a practical loss. A moderate number of hyaline glomeruli. The lymphatics around much engorged veins are much distended and they contain numerous lymphoid cells. These rather conspicuous areas are strictly localized and occur occasionally close to the capsule of Malpighian corpuscle. The stretched interstitial tissue of such parts is apt to carry a

moderate number of spindle cells. The intertubular and circumcapsular tissue is very generally edematous. The larger blood vessels are engorged, their adventitia thickened and edematous, their dependent lymphatics dilated and with numerous lymphoid cells. Spaces between the convoluted tubules are as a rule not stretched, but there exists considerable edema and engorgement around the Malpighian corpuscles of Bowman's capsule leading to scalloping and compression towards the tuft. The collecting tubules in loops contain hyaline casts. There exists occasional hyaline transformation or a hemorrhagic disintegration of the glomerulus but no attachment to its capsule. In the medulla exists marked streaky engorgement of the intertubular capillaries even to ectasy with edema and stretching of the interstitial substance, but no cellular infiltration or formation of new connective tissue. The larger vessels are considerably thickened in adventitia, but not in the intima.

Case No. 12. — No. 1715. A. M., female, 40 years, 107 pounds. Died Feb. 20, 1910; autopsied Feb. 21, 1910. Mitral stenosis, dilatation of the right heart, hydrothorax, general venous stasis. Heart weight, 350 grams. Right side collapsed, left contracted. The mitral valve scarcely admits tip of the finger, due to fusion of the cusps forming a slit-like band stretched across the opening. Left ventricle, 10 millimeters. Heart muscle shows brown atrophy. Left kidney, 150 grams. Dark in color. The capsule strips easily, exposing a somewhat pitted surface. The cortex thinner than normal, the markings much accentuated, vessels moderately thickened. Right kidney, 150 grams, like the left.

Microscopically: Malpighian corpuscles large. Tuft fills most of the capsule. Very marked engorgement of the tuft, sometimes transforming capillary loops to large sinuses. The epithelium of the glomerulus completely lost. The capsular wall, hyaline and edematous, shows in places fibrillar swelling, in others fibrillar separation. The pericapsular tissue edematous. The edematous intertubular tissue carries narrow compressed rows of fibroblastic cells rather compressed between edematous tubules. Streaky and patchy thickening around blood vessels and dilated lymphatics in the neighborhood of and between Malpighian corpuscles. Most of the thickening here appears also to be due to edematous swelling of the connective tissue and obstruction of lymphatics around the blood vessels. Malpighian corpuscles show generally no hyaline transformation or adhesion to the capsular epithelium. Occasionally enormously dilated lymph spaces with coagulated lymph adjoin a Malpighian corpuscle. The convoluted tubules at some slight distance from the glomeruli appear intact and the intertubular tissue not swollen and not thickened, without fibroblasts, only engorged. In other parts the kidney shows only evidences of pure but very marked stasis without much edema or swelling of tissue around glomeruli and blood vessels. In the cortex the latter are thickened and there exist few infarcts around thrombosed obliterated arteries. In the medulla the vessels are very thick. There exists marked general, sometimes tremendous, engorgement of the intertubular vessels to ectasy

and interstitial edema. The intertubular tissue is much stretched, but not quantitatively increased. Fibroblasts appear only in isolated spots. There are many hyaline casts in the collecting tubules which appear to be formed by a direct transudation from the lymph spaces through cells into tubules. Occasionally they contain a desquamated epithelial cell or enclose remnants of blood pigment, while the neighborhood may show some free red cells.

Case No. 13. — No. 1760. B. K., male, 60 years, 165 centimeters, 132 pounds. Died March 23, 1910; autopsied March 26, 1910. Marked fatty infiltration of the heart. Weight, 500 grams. The tricuspid admits three fingers. The mitral over two fingers. There is no valvular lesion, but the heart muscle is flabby, friable, greasy, and pale brown in color. Lungs emphysematous.

Left kidney, 250 grams, large, dark in color. On section the capsule strips easily, the surface is smooth, the stellate veins deeply injected. The cortex of good proportions, the medullary rays well marked; glomeruli also. The vessels moderately thickened. The right kidney, 200 grams, corresponds to the left.

Microscopically: The Malpighian corpuscles mostly large. The tuft very markedly engorged fills capsule either completely or at least to a great extent, in which case the capsular space shows albuminous coagulum or few blood cells. The epithelium of the tuft is lost. The capillary loops of the tuft are sometimes dilated to the extent of sinuses. Again, in a number, by fusion and hyaline transformation (pressure and fusion with transudate), they shrink to hyaline globules attached to the epithelium of the capsular wall. The latter in the distended corpuscles is usually thin and fibrillar. Occasionally the tuft is entirely lost and there remains only a homogeneous coagulated mass surrounded by a dilated capsule. A gradual compression and collapse of the loops of the tuft, probably due to pressure from without (and nutritional disturbances), may be seen commencing in some glomeruli at the periphery of the tuft and progressing towards the afferent vessels. This leads to collapse of the capsule. In some glomeruli the afferent vessel appears decidedly thickened. There also occurs occasional hemorrhagic disintegration of the glomerulus. The convoluted tubules are large and well preserved, the intertubular tissue wide with edematous stretching, but not thickened. The cells of the convoluted tubules on the whole are well preserved, although in some tubules they show granular disintegration and desquamation and there exists very evident and abundant formation of new nuclei and multinuclear cells. The capillaries very widely dilated, in places truly ectatic. There are few casts in the collecting tubules. The larger vessels show a sometimes enormous dilatation. Their walls thickened, in few places lymphoid cells are numerous in the accompanying dilated lymphatics, but generally one sees no thickening due to new connective tissue formation or no cellular infiltration around glomeruli or tubules. The collecting tubules and loops contain blood with a hyaline transformation of red cells into

casts. In the neighborhood of a few larger, markedly engorged vessels there can be noticed some localized tissue thickening with infiltration of lymphoid cells in the tissue spaces around Malpighian corpuscles and between the tubules. The loops and the collecting tubules show prominently the formation of new cuboidal cells which are sometimes multinuclear. The thickened tissue is unlike the ordinary connective tissue, but made up of the network of fibrils previously described and probably derived from the old connective tissue fibers and transuded or edematous fluid. It contains no or few old spindle cells. There exists therefore no appreciable formation of new connective tissue.

Case No. 14. — No. 1769. M. L., male, 74 years, 165 centimeters, 111 pounds. Died Jan. 3, 1910; autopsied Jan. 4, 1910. Carcinoma of esophagus, mitral and aortic sclerosis. Heart weighs 375 grams. Dilated, filled with clots. The tricuspid and mitral valves admit three fingers. Left ventricle measures 10 to 12 millimeters. Musculature friable, brownish red, fatty. Extreme emphysema of both lungs. General stasis.

Left kidney, 150 grams. Dark reddish. Vessels much injected. Capsule strips easily. Surface smooth, with few small cysts. Vessels at base of pyramids thickened. Markings of the kidney substance distinct. Cortex narrow. Right kidney, 150 grams, like the left.

Microscopically: Malpighian corpuscles generally large. The tuft fills the capsule, is engorged. In some of them obliteration of the loops by hyaline swelling and fusion of the endothelium of the capillaries followed by collapse of glomerulus evidently due to block in circulation. An occasional completely hyaline Malpighian corpuscle. Epithelium of the glomerulus lost. The epithelium of the capsule swollen and turbid. Generally there is not found adhesion of the capsule and only few show this with the following hyaline transformation. No appreciable connective tissue thickening or cellular infiltration around the capsule except in the neighborhood of larger thicker vessels where there is some increase in fibroblastic cells involving the neighboring capsules. Irregular but marked engorgement of the intertubular capillaries in isolated spots, the appearance of limited fibroblasts compressed between well preserved convoluted tubules. A moderate desquamation of tubular cells exists and a moderate formation of new nuclei leading to multinuclear cells particularly in the convoluted tubules. Medulla: Capillaries show marked engorgement; very marked dilatation of lymphatics, edema, and stretching of interstitial tissue. The medullary tubular parts are generally intact, — no appreciable formation of casts. The walls of the larger vessels show essentially adventitial thickening.

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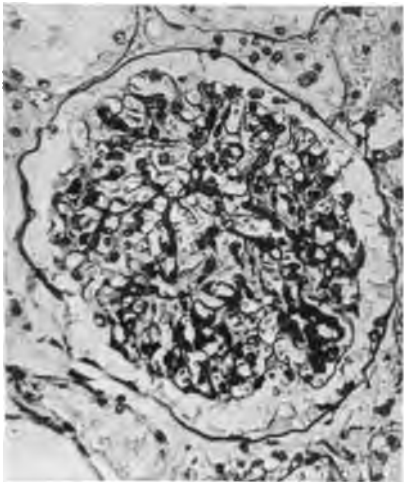
EXPLANATION OF PLATE XVI.

FIG. 1. — Zeiss; magnification 150. Glomerulus in advanced moderate stasis. Hyaline, scalloped capsule with atrophic lining epithelium. Tuft engorged. Reflected epithelium largely lost, leaving naked capillaries; capillary endothelium compressed, and capsular space contains some coagulum.

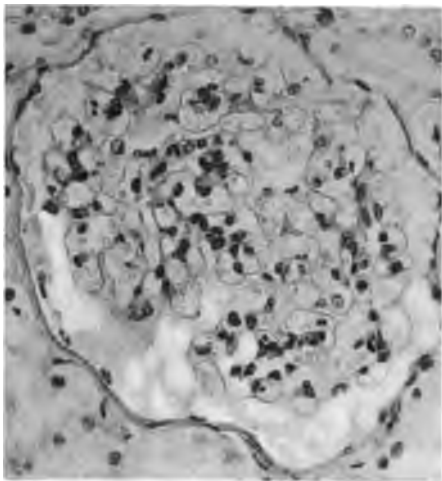
FIG. 2. — Zeiss; magnification 350. Glomerulus in advanced marked stasis. Capsule swollen, hyaline, scalloped. Reflected epithelium over the tuft entirely lost, leaving the massively engorged capillaries bare. Their endothelium much compressed, thin. Capsular spaces contain granular coagulum and detritus.

FIG. 3. Zeiss; magnification 350. Transudate cast in a collecting tubule. Towards the upper left hand (from observer) cast continuous with tubular wall and passes over and between lining cells to surrounding lymphatic. Cellular detritus (red blood cells and an epithelial cell) fusing to cast in lumen of a loop in upper right hand.

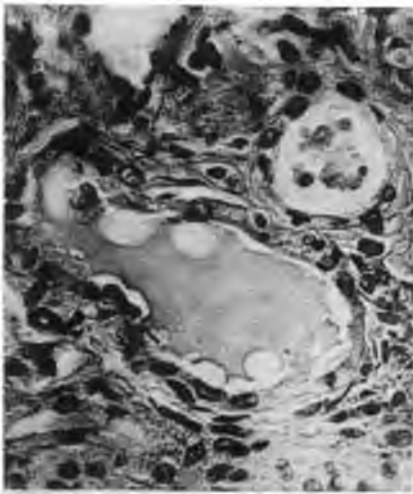
FIG. 4. — Zeiss; magnification 150. Medulla from advanced stasis. Engorgement of capillaries, much edematous interstitial stretching and separation of tubules. Collecting tubules and loops show abundant formation of new nuclei and multinuclear cells lining the tubular walls only.



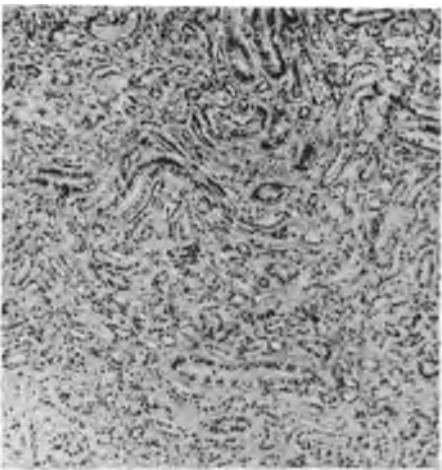
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Oertel

Cyanotic Kidney

THE ACTION OF CERTAIN PRODUCTS OBTAINED FROM THE
TUBERCLE BACILLUS.*

A. *Cleavage Products of Tuberculo-protein obtained by the Method
of Vaughan. Communication I.*

THE POISONOUS SUBSTANCE.

BENJAMIN WHITE AND OSWALD T. AVERY.

(From the Department of Bacteriology, Hoagland Laboratory, Brooklyn, N.Y.,
and the Saranac Laboratory for the Study of Tuberculosis.)

SYNOPSIS.

- I. INTRODUCTORY.
- II. PREPARATION OF MATERIAL (cleavage products from tuberculo-protein).
- III. ACTION OF THE POISONOUS SUBSTANCE:
 - 1, Technic of injections; 2, Symptoms and gross pathology; 3, Titer of fatal dose; 4, Toxicity of dried tubercle bacilli and toxicity of extracted tubercle bacilli; 5, Effect of heat on toxicity of the poison; 6, Effect of poison on body temperature; 7, Does the poisonous substance contain any sensitizing groups? 8, Does the poison immunize to itself? 9, Absorption of the poison with brain tissue; 10, Absorption with lung tissue; 11, Absorption with liver tissue; 12, Action of complement on the poison; 13, Skin tests.
- IV. THE PROTECTIVE EFFECTS OF VARIOUS SUBSTANCES:
 - 1, Atropin; 2, Morphia; 3, Chloral; 4, Lecithin.
- V. SUMMARY.
- VI. BIBLIOGRAPHY.

I. INTRODUCTORY.

In the present state of our knowledge of protein poisons and hypersensitiveness the more prominent problems inviting solution are:

1. The relation between the chemical composition and the vital reactions of the substances causing hypersensitiveness, and
2. The relation of hypersensitiveness to bacterial infection.

It is now well established that the parenteral and, under

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suitable conditions, also the enteral introduction of protein renders an animal highly sensitive to a second similar introduction of the same substance. There are already much data concerning the specific nature of the reaction and the amounts of protein necessary to sensitize and to intoxicate. There has been repeated and apparently thorough investigation of such problems as, (1), the interval elapsing between the initial introduction of antigen and the appearance of sensitiveness; (2), the dosage of; (3), and interval between repeated injections necessary for the production of increased hypersensitiveness; (4), the duration of this condition; (5), its inheritance, and (6), the mechanism of its transference to normal animals. Any study of these questions promises to yield an interpolation of details rather than the disclosure of any new conceptions in this new phase of immunity. As regards the physiology and pathology of hypersensitiveness, more has been learned of the nature of the intoxicating shock than of sensitization proper; the effects rather than the causes have been analyzed. For the effects we have theories — theories not yet established beyond criticism — while for the causes we have only hypotheses. These aspects, however, are more or less collateral to or dependent upon a larger chemical problem, and they might well be considered from this viewpoint. It would therefore seem that greater gains toward a full knowledge of anaphylaxis would accrue from efforts directed toward the solution of the chemical nature of the phenomena of anaphylaxis and the relation of hypersensitiveness to infection.

It seems to be definitely accepted so far that proteins are the sole substances capable of inducing the condition now known as anaphylaxis, or perhaps more properly as hypersensitiveness. It also seems to have been shown that, in order to intoxicate a protein sensitized animal, the identical or intimately related protein is necessary. On the other hand, repeated observations show that certain proteins, and protein derivatives as well, possess an intrinsic ability to produce in normal animals a toxic condition which bears

the closest resemblance to the intoxication shock in hypersensitive animals. The conditions so similar to anaphylaxis brought about by the injection of some heterologous sera, by peptones and other degraded proteins in untreated animals are illustrations of this primary toxic action. The study of the chemistry of proteins and protein derivatives with special reference to their physiological action would appear to offer promise of affording an analysis of the phenomena of hypersensitiveness. The questions might be stated thus: (1), Is the intact molecule essential to the production of hypersensitiveness, or does the molecule contain a sensitizing moiety combined with non-essential atom groups? (2), Is the whole molecule necessary for intoxicating a hypersensitive animal, or does the molecule possess an intoxicating increment combined with a sensitizing group? and (3), Does the protein molecule possess a moiety or moieties capable of intoxicating a normal animal?

Vaughan was the first to study the chemistry of the protein molecule in relation to its immunizing, sensitizing, and intoxicating properties. By the aid of an alcoholic solution of sodium hydrate he effected a cleavage of protein into two fractions — both retaining, in part, their protein nature. One of these fractions, the alcohol soluble portion, was poisonous but non-sensitizing ("toxophore"), while the other — the alcohol insoluble residue — was sensitizing but non-poisonous ("haptophore"). As might be expected, all proteins studied were found to yield sensitizing portions, but it was quite unexpected to find that all proteins, whether primarily toxic or not, yielded a poisonous fraction. What was still more strange, all of these poisonous fractions appeared to be identical in their composition and effects. Such innocuous substances as egg albumin, serum proteins, and proteins from non-pathogenic bacteria, yielded a poison quite as potent as did the proteins from virulent pathogenic bacteria. Vaughan therefore concludes that all proteins possess a common poisonous group, and, on the other hand, all proteins possess a sensitizing group which is peculiar to that particular protein from which it is derived.

Much of the criticism directed against Vaughan's work has been largely academic in character and is based on an *a priori* consideration of the methods he employed rather than on actual experiments. The chief objection raised has been that the process of obtaining split-protein products, *i.e.*, by boiling with alcoholic sodium hydrate, constitutes a radical chemical procedure, and that, therefore, the substances produced by this hydrolysis are artificial products and not true cleavage derivatives. This objection is not necessarily valid. In order to obtain from proteins, by artificial means, the various substances of decreasingly smaller molecules resulting normally from enzymatic digestion *in vivo*, it is necessary to submit the protein to a more or less violent chemical attack. Such substances arising from a sufficiently prolonged hydrolysis by means of acid, or in an autoclave, differ in quantity perhaps but not in kind from the substances resulting in pancreatic digestion, and their production can be controlled by regulating the acceleration and intensity of the cleavage. It has been further contended that the amount of the isolated poisonous fraction required to produce a fatal intoxication in a normal animal is in excess of the amount of poison that would arise from the vital splitting of the intoxicating dose of protein in a hypersensitive animal. This contention similarly applies to the sensitizing portion, as it apparently requires a relatively larger quantity of the cleavage residue to sensitize than of the original whole protein. This contention admits of some discussion. The alcohol soluble fraction obtained by Vaughan's method probably contains, after neutralization and removal of the salt, several substances representing very closely allied degradation stages. Further, halting the hydrolysis at a given point would not necessarily yield the whole poisonous moiety of the original mother molecule, since at any given stage, in addition to the yield of the particular poisonous substance or substances formed, there would be higher complexes not yet split to the poisonous stage, as well as a certain amount of poison which had passed on to simpler derivatives. The amount of such accompanying products

in a particular lot of the alcohol soluble fraction would naturally depend upon the velocity of the hydrolysis. The poisonous substance, or so-called "Toxophore," is not to be considered as a definite chemical entity, but as an unknown substance mixed with certain closely allied chemical bodies which are more or less irrelevant. Therefore it must be emphasized that the amount of toxophore obtained from any given amount of protein by no means represents the total potential toxic moiety of that protein. Such an explanation applies equally well to the sensitizing cell residue.

For one familiar with Vaughan's work there can be no doubt that he has succeeded in isolating from the cleavage of bacterial and other proteins two substances which possess exceedingly interesting chemical and physiological properties. The exact chemical nature of these two substances is yet to be determined. By the reactions they give, both show that they still retain some of their original protein characteristics. By the effects they produce in the animal body it would seem that they individually represent the sensitizing and intoxicating moieties originally combined in the whole protein molecule. In the present communication only the poisonous fraction will be considered.

Vaughan found that this fraction, when injected into the peritoneal cavity of a guinea-pig, apparently produced all the symptoms of acute anaphylactic intoxication. The question at once arises, are these two conditions identical, and, if so, what is the relation of this substance to the theoretical anaphylatoxin liberated from protein in the sensitized animal body, or in the test-tube by serum, as claimed by Friedberger? If this identity can be established, then, in this poisonous fraction may be found the means of solving many of the problems of anaphylaxis.

That a comparatively simple protein derivative may produce acute fatal shock is by no means an isolated or particularly recent observation. In this connection the studies of Schmidt-Mülheim,¹ published in 1880, of Pick and Spiro,²

Underhill,¹⁷ and others, on the physiological action of peptone, take on a new significance. The physiological chemistry of protein digestion products has naturally received much attention and there is considerable detailed information concerning their various actions upon the blood, lymph, and nervous systems. These earlier studies, with the addition of Thompson's³ investigations on the action of protamines and histones, would appear to afford a basis for analyzing some of the phenomena of anaphylactic intoxication. In their newer aspects these protein derivatives have been studied by Popielski,⁴ Biedl and Kraus,⁵ and Schittenhelm and Weichardt.^{6,7} The whole subject has been reviewed recently by Schittenhelm,⁸ in which review fuller details may be sought. To briefly summarize his conclusions (and his conclusions are based both upon the experience of others and upon his own results) it has been shown that bacterial proteins, protamines, histones, and the more complex peptones are primarily toxic, while native proteins such as the albumins, globulins, fibrins, and nuclealbumins, and the lower peptones and amino acids, are relatively non-toxic. The reason for the toxicity of bacterial proteins is not clear since whole proteins, with a few exceptions such as ricin and abrin, produce signs of a poisonous action only when administered in amounts sufficient to cause mechanical or osmotic disturbances.

The physiological action of such protein fragments as peptone, protamines, and histone resembles in so many respects the intoxication in anaphylaxis that it seems reasonable to associate them, and, reasoning by analogy, to see in their action support for the theory that anaphylactic shock is due to a parenteral digestion of protein.

It was the purpose of the present investigation to study in some detail, and with special reference to anaphylaxis, the physiological action of the poisonous substance obtained from tuberculo-protein by the method of Vaughan. The plan includes the repetition of many of Vaughan's experiments and those of Banzhaf and Steinhardt,⁹ who employed cell poisons obtained by a similar method from various sources. The principal variation between their work and

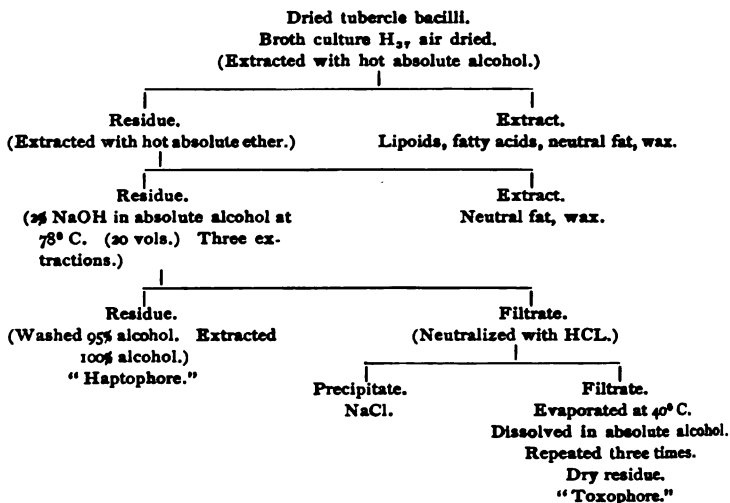
the present research consists in certain modifications of technic and the addition of some experiments, which, it was felt, would throw additional light on the nature of the action of the protein poison.

II. PREPARATION OF THE MATERIAL.

The dried bacterial mass used in the preparation of these products was obtained from cultures of *Bacillus tuberculosis*, typus humanis (Saranac Laboratory, Strain H₂₇). This strain is virulent for guinea-pigs. The surface growth from three to six weeks old glycerine broth cultures was collected on porcelain filters, drained and washed with water until the washings failed to show any trace of chlorides. The moist material was thoroughly dried over sulphuric acid in vacuo at room temperature. The dried bacilli (fifty grams) were then ground to a fine powder in a porcelain ball mill (about one hundred and fifty hours grinding). The powder was placed in a Soxhlet apparatus and extracted with absolute alcohol until the solvent gave no residue on evaporation. Extraction was then continued with absolute ether as above. Prolonged extraction with alcohol and ether even for several weeks failed to completely rob the bacilli of this strain of their acid-fastness. The extracted bacilli were then treated by the method of Vaughan. The dry powder was placed in a flask under a reflux condenser, twenty volumes of a two per cent solution of chemically pure sodium hydrate in absolute alcohol were added and boiled for one hour. The mixture was filtered hot, the residue being returned to the flask, boiled again for the same time with alcoholic sodium hydrate, filtered and the operation repeated a third time. The combined filtrates were exactly neutralized to litmus solution with hydrochloric acid, the resulting precipitate removed by filtration and the filtrates evaporated to dryness in vacuo, the temperature never rising above 45° C. The residue was taken up in warm absolute alcohol, filtered, and the filtrate evaporated as before. The operation was repeated three times and the final residue ground to a fine powder and kept over sulphuric acid. The preparation represents the protein poison (toxophore) and was designated as III. T.

The cell residue left after the thrice repeated boiling with alcoholic sodium hydrate was repeatedly washed with ninety-five per cent alcohol by decantation, then with absolute alcohol and finally extracted in a Soxhlet apparatus with absolute alcohol until the extract gave no residue and was neutral to litmus, then dried in vacuo. This constituted the sensitizing portion of the tuberculo-protein and was designated as III. H. (haptophore) and will be dealt with in another communication.

PREPARATION OF SPLIT PRODUCTS OF TUBERCLE BACILLI. (VAUGHAN.)



The alcohol soluble portion, the toxophore (Preparation III. T.), is a yellowish-brown powder with a characteristic pungent odor. It is readily soluble in absolute alcohol, giving a clear solution. It dissolves in water and in physiological salt solution, giving a slightly opalescent fluid, faintly acid to litmus. The faint turbidity increases on heating but disappears on cooling. The watery solution gives a distinct biuret test. The powder gives a faintly cloudy solution, with hot concentrated nitric acid which clears, then becomes a strong but not deep yellow upon the addition of ammonium or sodium hydrate. The Adamkiewicz and Liebermann reactions are both positive, while Millon and Molisch are faintly positive. The addition of bromine water to a watery solution of the powder produces a white flocculent precipitate but no color, showing the absence of tryptophane.

In the present instance no attempt has been made to investigate the chemical nature of this tuberculo-poison, although it is planned to take up the matter in the near future. Vaughan and his pupils, particularly Wheeler,¹⁰ studied in detail the constitution of similar poisons derived from other proteins. The poison, as obtained by Vaughan's method, is undoubtedly crude. Its various reactions and its behavior in different solvents show it to be a mixture of several substances, some of which may be removed without decreasing its toxicity. It is probable that the poisonous part of the powder may be composed of several substances,

closely allied through their chemical and physiological properties. All the reactions of this poisonous fraction argue for its protein character, but its exact nature and constitution are yet to be determined.

III. THE ACTION OF THE POISONOUS SUBSTANCE.

1. Technic of injections:

All guinea-pigs were from the same source and were as near two hundred grams in weight as it was possible to obtain them. The dry poisonous substance was dissolved in sterile .9 per cent salt solution in the proportion of .010 gram poison in one cubic centimeter. The injections were made, unless otherwise noted, in one of the external jugular veins. Owing to anatomical differences and operative convenience the injection is usually more successful when the right jugular is used. Autopsies were performed immediately after death.

2. Symptoms and gross pathology:

In the present experiments the effects noted after the intravenous injection of the poison are striking. When a quantity approximating the minimum fatal dose is given, the first symptoms appear immediately, or, at most, within thirty seconds. The animal becomes restless, scratches its nose, and frequently utters a sharp hiccough. The movements become incoördinate, the gait is unsteady. The eyes are fixed, and stare. Respiratory embarrassment with diaphragmatic spasm sets in and increases to a degree which causes the animal to spring from its feet, to buck, and finally to fall on its side with convulsive twitching of its legs, intermittent, and both clonic and tonic in character. Involuntary micturition and defecation frequently take place. The dyspnea becomes more marked, and then ensue successive periods of apnea lasting as long as twenty to thirty seconds. These are followed by violent inspiratory efforts, during which the chest wall becomes fixed in maximum inspiration. Cyanosis is noticeable in the lips and ears and becomes more marked. The convulsive gasps increase in frequency and decrease in depth, until finally only the lips move, the feeble and rapid dilatations of the *alæ nasi* marking the onset of death. This sequence of symptoms is accompanied by a rapid and progressive fall of the body temperature. Death takes place in from one and one-half to six or seven

minutes. Immediate autopsy reveals first a cyanotic hue of the subcutaneous and muscular tissues. The blood is dark in color and does not clot readily. Beyond an exaggerated peristaltic movement of the intestines, the abdominal viscera appear to be normal. On opening the chest the lungs are found to be in a state of maximum inflation, overlapping the precordium, and forming a cast of the thoracic cavity. They are pale and often slightly bluish in color and frequently exhibit punctate hemorrhages on the surface. The heart still beats. Not infrequently there is definite heart block, with an auriculo-ventricular arrhythmia of three to one. Often there are petechial hemorrhages in the epicardium. Greater extravasations are also seen, and in two cases actual rupture of the ventricle had apparently taken place. On section the lungs do not collapse and on pressure only a little frothy serum exudes. They are not edematous. They float on water. The excised heart continues to beat for several minutes. The gross appearance of the brain is normal. A study of the pathological changes in the histology of the lungs, heart, and brain has been undertaken but has not yet progressed sufficiently to warrant any conclusions. When the dose is larger the acute symptoms appear instantaneously and their sequence is more rapid. With a sub-lethal dose the onset is slower and the manifestations are less violent. The animal shows evidence of weakness, drags its hind legs, and frequently lies on its side in collapse. The apneac stage is never reached, its appearance therefore signifies inevitable death. Recovery from a non-fatal dose is comparatively prompt even when near the lethal border line. Recovered animals exhibit no visible sequelæ of the intoxication.

During the present investigation there has been frequent occasion to compare the acute intoxication produced in normal animals by the tuberculo-poison with the fatal shock in hypersensitive pigs following the intravenous injection of the specific protein. It has been found impossible to discover any appreciable points of difference in the symptomatology and gross pathology of the two conditions. They would therefore appear to be identical.

3. Determination of minimum lethal dose:

(COMBINING EXPERIMENTS I, 8, 11, 12, 13.)

Pig.	Weight.	Preparation.	Dose (grams).	NaCl (cc.).	Proportion to Body Weight.	Result.	Autopsy.
45 . .	190	II. T.*	0.019	1.90	1:10000	xxx D. 5' 32''†	Typical.
38 . .	180	III. T.	0.018	1.80	1:10000	xxx D. 3' 30''	"
39 . .	190	II. T.	0.015	1.50	1:12000	xxx D. 4'	"
40 . .	190	III. T.	0.0159	1.59	1:12000	xxx D. 3' 30''	"
193 . .	215	"	0.0180	1.8	1:12000	xxx D. 5'	"
31 . .	320	"	0.025	0.50	1:12800	xxx D. 6'	"
41 . .	200	"	0.0143	1.43	1:14000	xxx D. 3' 15''	"
192 . .	190	"	0.0127	1.27	1:15000	xxx D. 4' 30''	"
290 . .	210	"	0.014	1.4	1:15000	xxx D. 4' 30''	"
291 . .	215	"	0.0143	1.43	1:15000	xxx D. 4'	"
292 . .	225	"	0.015	1.5	1:15000	xxx D. 4' 15''	"
36 . .	320	"	0.020	2.5	1:16000	xx R.	—
19 . .	405	"	0.025	2.0	1:16200	xx R.	—
5 . . .	350	II. T.	0.020	4.0	1:17500	xxx R.	—
44 . .	225	"	0.0125	1.25	1:18000	xxx D. 3' 40''	Typical.
42 . .	205	III. T.	0.0114	1.14	1:18000	xxx D. 3' 20''	"
35 . .	370	"	0.020	2.0	1:18500	xx R.	—
98 . .	190	II. T.	0.0086	0.86	1:22000	xx R.	—
43 . .	205	III. T.	0.0082	0.82	1:25000	x R.	—
32 . .	330	"	0.010	0.2	1:33000	xx R.	—
20 . .	385	"	0.0125	1.0	1:38000	x R.	—

* II. T. prepared the same as III. T.

† x R. Slight symptoms—spasmodic cough—bucking—recovery.

xx R. Marked symptoms—spasmodic cough—bucking—respiratory distress—recovery.

xxx D. Fatal—very acute—fulminating.

In addition, eight other pigs varying in weight from three hundred to four hundred and thirty grams were given doses of from 1:12000 to 1:4000, which resulted in death in seven minutes or less. From the above it was determined that one part of dry poison to fifteen thousand parts of body weight represents the minimum fatal dose for animals weighing not more than two hundred and fifty grams. Undoubtedly, further purification by re-solution, filtration and evaporation

would increase the toxicity of the preparation, but the incidental loss of material was too great to make it practicable in the present instance.

4. Toxicity of pulverized dry tubercle bacilli and of the same extracted with alcohol and ether:

For purposes of comparison it was deemed desirable to observe the toxic effect of injections of dried tubercle bacilli and of the same bacilli after being thoroughly extracted with boiling alcohol and then ether. It might be noted here that the material was found to be non-infectious. The dry powder was emulsified in physiological salt solution and injected intraperitoneally.

EXPERIMENT 2. (Dried tubercle bacilli.)

Pig.	Weight.	Dried Tubercle Bacilli (grams).	Relation to Body Weight.	Result.
25.....	295	0.85	1 : 250	Death 22 hours.
26.....	285	0.57	1 : 500	" 21 "
27.....	275	0.275	1 : 1000	" 24 "
13.....	375	0.188	1 : 2000	Recovery.
3.....	390	0.13	1 : 3000	Death 36 hours.
14.....	355	0.118	1 : 3000	Recovery.
15.....	345	0.086	1 : 4000	"
16.....	305	0.06	1 : 5000	"
17.....	255	0.026	1 : 10000	"

EXPERIMENT 5. (Alcohol-ether extracted tubercle bacilli.)

Pig.	Weight.	III. R.* (grams).	Relation to Body Weight.	Result.
21.....	400	1.60	1 : 250	Death 24 hours.
22.....	350	0.70	1 : 500	Death 30-40 hours.
23.....	340	0.34	1 : 1000	Recovery.
10.....	300	0.3	1 : 1000	Death 26 hours.
11.....	280	0.112	1 : 2500	Recovery.
12.....	275	0.07	1 : 4000	"
8.....	320	0.064	1 : 5000	"
9.....	270	0.027	1 : 10000	"

* III. R. represents the tubercle bacilli after extraction as described.

These intraperitoneal injections called forth no acute symptoms. After an hour the animals appeared sick, trembled, and showed symptoms of a slow poisoning. Autopsy showed a plastic fibrinous peritoneal exudate with visceral congestion. Krause has found that as much as twenty-five cubic centimeters of watery extract representing .250 gram of the dried substance of the tubercle bacillus may be injected intravenously in normal pigs without eliciting any anaphylactic symptoms.*

* Personal communication.

5. Effect of heat on the toxicity of the poison :

The dry powder was dissolved in physiological salt solution (.010 gram in ten cubic centimeters) and boiled over the free flame for one minute. Animals 99, 100, and 101 received a similar solution which had been filtered hot after boiling, the filtrate being again boiled and filtered.

Pig.	Weight.	Preparation.	Dose (grams).	NaCl (cc.).	Proportion to Body Weight.	Result.	Autopsy.
46 . .	230	II. T.	0.023	2.3	1 : 10000	xxx D. 3' 15"	Typical.
47 . .	240	III. T.	0.024	2.4	1 : 10000	xxx D. 4'	"
96 . .	195	"	0.0108	1.08	1 : 18000	xxx D. 4'	"
97 . .	200	"	0.008	0.8	1 : 25000	xx R.	—
99 . .	200	II. T.	0.0166	1.66	1 : 12000	xxx D. 5' 45"	Typical.
100 . .	200	"	0.015	1.50	1 : 13333	xxx D. 3'	"
101 . .	195	"	0.0108	1.08	1 : 18000	xx R.	—

All injections intravenous.

The poisonous substance is therefore thermostabile within the limits observed. This is in agreement with the observations of Ritz and Sachs,¹¹ who found the poisonous principle of peptone to be resistant to heat. The anaphylatoxin, however, is thermolabile. The slight precipitate appearing when the solution is heated is evidently non-toxic since its removal by filtration does not impair the original titer of the poison.

6. Effect of the poison on body temperature :

The question of the action on the heat-regulating mechanism is one of importance. Its bearing upon the production of fever in infectious diseases is obvious if such changes in temperature are to be looked upon as modified intoxications due to a hypersensitiveness to bacterial or somatic protein. Reasoning from analogy, it would be expected that small doses would elevate the temperature while excessive or fatal doses would depress it. Friedberger has observed that small intoxicating doses of protein produce an elevation of temperature in sensitive animals. Vaughan,¹² in a recent paper on protein fever, reports that the injection of comparatively

large but sub-lethal doses of the cleavage poison produces pyrexia in rabbits. He also found that frequently repeated injections of small amounts of protein gave rise to the same condition, and therefore he considers fever as a manifestation of hypersensitive intoxication. A fatal dose of protein in a sensitive animal invariably produces a rapid fall in temperature. The difference between fever and the acute drop in fatal anaphylactic collapse would therefore appear to be dependent upon quantitative rather than qualitative factors.

EXPERIMENT 39. (III-19-12.)

Fig.	III. T. (grams).	NaCl (cc.).	Site of Injection.	Fore Period.	Time Injected.	15 Min.	30 Min.	60 Min.	2 Hrs.	3 Hrs.	4 Hrs.
301 ...	0.010	1.0	Subcutaneously.	10:07 39.9	10:32	39.2	39.0	39.5	39.9	39.5	39.5
310 ...	0.010	1.0	"	10:10 39.6	10:34	39.6	39.3	39.5	39.6	39.7	39.4
311 ...	0.010	1.0	"	10:12 39.9	10:36	39.6	39.5	39.7	39.6	39.7	39.4
312 ...	0.010	1.0	"	10:14 39.7	10:38	39.6	39.6	39.6	39.6	39.4	39.5
303 ...	0.010	1.0	Intraperitoneally.	10:16 39.6	10:39	39.1	38.9	39.6	40.4	39.8	40.8
304 ...	0.010	1.0	"	10:18 39.5	10:40	39.0	38.6	38.3	38.9	39.1	39.4
313 ...	0.010	1.0	"	10:22 39.4	10:41	38.9	38.6	38.7	38.8	39.4	39.5
307 ...	0.010	1.0	"	10:24 39.6	10:42	39.6	39.6	39.6	40.3	40.3	40.5
308 ...	—	1.0	Subcutaneously.	10:26 39.3	10:31	39.2	39.2	39.2	39.1	39.2	39.2
309 ...	—	1.0	Intraperitoneally.	10:28 39.2	10:30	38.9	39.0	39.1	39.5	39.7	39.5

The animals used in Experiment 39 had previously received, at intervals of five days or more, intraperitoneal or subcutaneous injections of the poison in increasing amounts in order to determine the threshold of febrile reaction. The doses were .000001, .00001, .0001, and .001 gram respectively. The resulting variations in the body temperature were too slight and inconstant to have any significance.

One would expect that, if the poisonous fraction were concerned in fever production, a dose of .010 gram should elicit some indication of such action. The results recorded in the above table, however, are more or less negative. There appears to be a slight initial depression in the temperature followed by a corresponding elevation. There is certainly no fever production. It will be necessary to repeat this experiment using still larger doses and also repeating the injections at frequent intervals. The paucity of material makes this impossible at present.

7. Does the poisonous substance contain any sensitizing groups?

If these protein cleavage products are to be considered as representing the analogues of the sensitizing and intoxicating groups concerned in the phenomena of hypersensitiveness, then it must be shown that the cleavage effected by this method is sharp and complete. The cell-poison should be inert in producing sensitization and the cell residue should produce no signs of intoxication in either normal or specifically sensitized animals.

It was found desirable to defer the study of this phase of the subject to a more convenient occasion. The following table is inserted in the way of a preliminary note, without venturing any inferences from the result. The intoxicating dose of extract emulsion had repeatedly been found sufficient to kill properly sensitized animals. Pigs 52 and 53 showed no signs of intoxication, but a larger amount of extract emulsion would have been desirable. It may be also noted here that the cell residue as prepared above renders animals sensitive to whole tuberculo-protein, but in doses as large as .500 gram it fails to intoxicate either sensitive or normal animals.

EXPERIMENT 15.

Fig.	Weight. X-24 (grams).	X-24 1:5000 (grams).	X-30 1:4000 (grams).	X1-6 1:3000 (grams).	X1-13 1:2000 (grams).	X1-28 1:1500 (grams).	X1-29 1:1000 (grams).	X11-12.		Result.	Autopsy.
								Weight (grams).	Dose (grams).		
52.....	270	0.0054	0.00675	0.009	0.0135	0.018	0.027	310	0.75 cc. E.E.	O R.*	—
53.....	310	0.0062	0.00778	0.0103	0.0155	0.0207	0.031	0.75 cc. E.E.	O R.	—

* O R. = No anaphylactic symptoms — recovery.

The injections were all intraperitoneal with the exception of those on X-30 (subcutaneous) and the final intoxicating injection (intravenous).
The Extract Emulsion was prepared according to the method of Krause.

8. Does the poison immunize to itself?

Vaughan found that repeated intraperitoneal injections of protein poison render rabbits and guinea-pigs somewhat more resistant to its action. He concludes that the poisonous group produces no antitoxic antibodies in animals thus treated. The lessened susceptibility would seem to be due more to an acquired tolerance than to a true immunity.

EXPERIMENT 15.

Fig.	Weight X-24 (grams).	X-24 1:5000 (grams).	X-30 1:4000 (grams).	X1-6 1:3000 (grams).	X1-13 1:2000 (grams).	X1-22 1:15000 (grams).	X1-29 1:10000 (grams).	X11-12.		Total (grams).	Result.	Autopsy.
								Weight (grams).	Dose (grams).			
Subcu- taneously	48.	0.0048	0.006	0.008	0.012	0.016	0.024	335	0.0293-1:12000	0.0798	xxx D. 4'	Typical lungs and hemor- rhages.
	49.	0.005	0.00695	0.0083	0.0125	0.0167	0.025	350	0.0295-1:14000	0.07375	xxx D. 3' 30"	
	50.	0.0055	0.00687	0.0091	0.01345	0.0183	0.0275	380	0.0211-1:18000	0.0865	xxx D. 6' 15"	
Intraperi- toneally	51.	0.005	0.00695*	0.0083	0.0125	0.0167	0.025	310	0.0141-1:22000	0.07375	xx R.	—

* Subcutaneous injection.

The animals treated as above all gained weight and showed no appreciable effects incident to the repeated injections. They failed to develop any changed reactivity to the poison. No immunity was produced. The difference between this poisonous substance and the true toxins is obvious.

The bearing which the refractory stage produced by the poisonous substance has upon the phenomenon of antianaphylaxis is a question of sufficient importance to merit individual consideration. It will accordingly be dealt with in another communication. The data obtained seem to show that the poison may take an important part in preventing a subsequent intoxication with the poison itself, or in true anaphylactic intoxication. One experiment might properly be included here, on account of the present interest attached to it as well as by way of presaging a future report on the subject.

EXPERIMENT 21.

Date.	Pig.	Weight.	1st Injection III. T.	Interval.	2d Injection III. T.	Symptoms.	Autopsy.
XI-15-11	144	255	1:15000	48 hours.	1:12000	O Recovery.	O
XI-15	145	215	1:13190	"	1:12000	O "	O
XI-15	146	215	1:15000	"	1:12000	O "	O
XII-30	236	205	1:15000	"	1:12000	x "	O
II-26-12*	250	210	0.015 gm. chloral	24 hours.	1:15000	xxx D. 4' 30"	Typical.
II-26-12*	251	200	0.015 " "	"	1:15000	xxx D. 4'	"
II-26-12*	252	225	0.015 " "	"	1:15000	xxx D. 4' 15"	"

* Intravenous injection.

The first three animals were protected from the first dose with .015 gram chloral hydrate. Two days later they successfully resisted a supra-lethal dose of 1:12000. The three controls showed that the chloral played no part in protecting against the second injection. Pig 236 had received a mixture of fresh guinea-pig serum and a fatal dose of the poison two days previously (Experiment 35). The intravenous injection of a large but non-fatal dose of poison apparently renders the animal at least temporarily refractory to a subsequent similar injection of what would normally be

a fatal dose. The degree and duration of this insusceptibility will be tested.

With a view to comparing the behavior of this protein poison to that of certain bacterial toxins and, further, with a view to inquiring into the localization of its action, the absorption power of some tissues was studied. The technic of Wassermann and Takaki's¹⁸ classic experiment with tetanus toxin was adopted and, with some modifications, was in detail as follows :

9. Absorption of poison with brain tissue :

Two guinea-pigs of two hundred and five and two hundred and thirty grams respectively were killed by bleeding. The brains were immediately removed and ground together to a homogeneous paste. Two and one-half grams of this paste were thoroughly emulsified in ten cubic centimeters of salt solution containing .100 gram poison and the emulsion incubated for one hour at 37.5° C. It was then centrifuged and the residue, after a sharp and complete separation of the supernatant fluid, was washed with ten cubic centimeters of salt solution and again centrifuged. The opalescent supernatants were combined and immediately used for injection. Variations in the procedure were tried. The poison was incubated with a centrifuged extract of the brain and also with the extracted residue. The results were the same and are tabulated below.

EXPERIMENT 30. (XII-18-11.)

Fig.	Weight.	III. T.	NaCl Extract.	Symptoms.	Autopsy.
198	200	1 : 12000	1.67	xxx D. 8' 30"	Typical.
196	220	1 : 12000	1.83	xxx D. 4' 30"	"
199	230	1 : 15000	1.53	xxx D. 3' 15"	"
197	200	1 : 15000	1.33	xxx D. 4' 30"	"
201	205	1 : 15000	1.37	xxx D. 4'	"
200	215	1 : 20000	1.08	x D. 1 hr. 15'	Normal.

In the case of Figs 196 and 197 the poison was mixed with brain emulsion and centrifuged without incubation. Pig 201 received a brain emulsion in which the toxophore had been incubated for one hour and forty minutes. The emulsion-toxophore mixture given to Figs 198, 199, and 200 had been incubated fifty minutes.

It is evident that there is a marked dissimilarity between the absorption affinity of brain tissue for tetanus and diphtheria toxin on the one hand and for the tuberculo-poison on the other. Contact with brain tissue, under the conditions noted, apparently fails to affect the titer of the poison.

10. Absorption with lung tissue:

If the action of the poison is peripheral it is conceivable that lung tissue might have a selective affinity for this substance. The technic was precisely the same as that employed with brain tissue.

EXPERIMENT 33.

Date.	Pig.	Weight.	III. T.	NaCl Extract.	Symptoms.	Autopsy.
XII-29-11 . .	228	185	1:15000	2.47	xxx Slow D. 11'	Lungs collapsed.
" . .	229	200	1:12000	3.33	xxx D. 5' 45"	Typical.
" . .	230	210	1:15000	2.80	xx D. 12 hours.	—
" . .	231	220	1:12000	3.67	xxx D. 9' 45"	Typical.
I-22-12 . . .	276	210	1:15000	2.80	xxx D. 3' 10"	"
" . . .	277	210	1:15000	2.80	xxx D. 1' 30"	"

Again there appears to be no absorption. Slight variations were noted, but they might be traced to obvious technical difficulties.

11. Absorption with liver tissue. (Method precisely as above):

EXPERIMENT 34.

Date.	Pig.	Weight.	III. T.	NaCl Extract (cc.).	Symptoms.	Autopsy.
XII-29-11 . . .	232	195	1:15000	2.6	xxx D. 3' 45"	Typical.
" . . .	233	220	1:15000	2.83	xx D. 36 hours.	—
" . . .	234	215	1:15000	2.87	x R.	—
" . . .	235	215	1:12000	3.6	xxx D. 2'	Typical.
I-23-12	279	210	1:15000	2.8	xx R.	—
" . . .	280	200	1:15000	2.66	xxx D. 2' 40"	Typical.
" . . .	281	225	1:12000	3.75	xxx D. 3' 10"	"
" . . .	282	210	1:15000	3.5	x D. 1 hour 18'	"

Here there is some evident lack of uniformity in the behavior of the pigs. Three of the eight animals recovered and more or less atypical symptoms were noted in several cases. Contact with tissue and its incidental extraction must introduce conflicting factors. The reaction produced in some of the pigs, however, evidences the feeble affinity between the tissues studied and the poison.

These three experiments seem to emphasize the absence of any possible identity of this protein fragment with the true toxins. The results, however, are in accord with the symptoms produced by the cell poison. Recovery from a sub-lethal dose is rapid and complete and this would imply that the contact between the body cells and the poison is transitory and non-destructive. It appears to be more like a fulminating irritation, and may result in an arrest of function due to a disturbance in the physical equilibrium of the cells affected.

12. Action of normal complement on the poison :

The question of a possible relationship between the cell-poison and the anaphylatoxin of Friedberger naturally presents itself. The thermostability of the former as compared to the lability of the latter would seem to exclude such a relationship. A further comparison might be obtained by studying the action of complement upon the cell-poison since the lability of the anaphylatoxin in the presence of complement is generally accepted. An experiment to this purpose was carried out as follows :

Five guinea-pigs varying in weight from two hundred and twenty to two hundred and sixty grams were bled by cardiocentesis. The serum was obtained by allowing the blood to clot (one hour at 37.5° C. then three hours on ice) and was centrifuged clear. It was kept on ice twenty-four hours. To each cubic centimeter was added .010 gram III. T. and incubated at 37.5° C. as below. A corresponding amount of serum alone was incubated for corresponding lengths of time and injected in control animals. Cultures showed the serum and serum-poison mixture to be sterile in each case.

EXPERIMENT 35.

Date.	Pig.	Weight.	III. T.	Serum (cc.).	Incubated.	Symptoms.	Autopsy.
XII-30-11.	236	205	1:15000	1.37	2 hours.	x R.	—
"	239	205	1:15000	1.37	7 "	xxx D. 4' 45"	Typical.
"	241	210	1:15000	1.40	24 "	xx R.	—
I-24-12.	283	175	1:15000	1.16	2 "	xxx D. 4' 30"	Typical.
"	284	200	1:12000	1.66	2 "	xxx D. 3' 50"	"
"	285	220	1:15000	1.46	6 "	x D. 12 hrs.	—
II-9-12.	286	200	1:15000	1.33	6 "	xxx D. 3' 30"	Typical.
"	287	200	1:15000	1.33	6 "	xxx D. 4' 30"	"
"	288	200	1:15000	1.33	24 "	x D. 27' 30"	Lungs partly inflated.
"	289	200	1:12000	1.66	24 "	xxx D. 5'	Typical.

The control pigs showed no symptoms, and all promptly recovered from the operation shock. While there is a slight decrease in the titer of the poison, yet it cannot be claimed that normal serum under the above conditions has any marked destructive effect on the poison. It might possibly be that in the process of preparing the poisonous fraction it in some way becomes stabilized, but such a guess would seem to be forcing the analogy.

13. Skin tests with the poison.

If intradermic reactions are due to the action upon the local cells of the poison liberated in the splitting of antigen by free or sessile antibodies present at the point of injection, then it might be expected that the artificially prepared poison could produce a local reaction in normal animals. Such a supposition seems warranted by the general reaction following the administration of large subcutaneous, intraperitoneal or intravenous doses. If a local receptor mechanism is necessary for the binding of the poison to the dermal cells, then the poison should produce no inflammation. It is also possible that, all receptor interposition being excluded,

the poison is intrinsically inert in so far as the production of this particular manifestation is concerned.

Four series of pigs received intradermic injections of .00005 gram III. T. in .05 cubic centimeter physiological salt solution, with proper controls. The series included four normal animals, four which had been repeatedly treated with subcutaneous and intraperitoneal injections of the poisonous fraction (Experiment 13), four animals sensitized nineteen days previously with the cell residue and which had been found to be sensitive to an extract-emulsion of tubercle bacilli, and four animals rendered and proved sensitive by a watery extract of tubercle bacilli. The animals were closely observed and in no case was even a faint sign of a reaction noticed. It was impossible to similarly test tuberculous pigs, but this is shortly to be carried out.

IV. THE PROTECTIVE EFFECTS OF VARIOUS SUBSTANCES.

To obtain further light upon the possible parallelism between the fatal intoxication produced by the poisonous cleavage product and anaphylactic shock, it seemed desirable to study the possible protective action against the effects of the poison exerted by such substances as have been found to inhibit specific protein intoxication. Such a study might also afford evidence concerning the physiology of the reaction. The following experiments, however, are submitted with no intention of explaining the nature of the intoxication but merely for the purpose of recording comparative features of the two conditions.

1. The protective effect of atropine sulphate:

Auer and Lewis¹⁴ were the first to show that atropine exerts a prophylactic action upon anaphylaxis in the guinea-pig. The injection of .002 gram of atropine sulphate saved seventy-two per cent of their animals while seventy-five per cent of the controls died. Anderson and Schultz¹⁵ report the recovery of twenty-eight per cent of the animals which

had received atropine just previously to the intoxicating injection of protein. Mita,¹⁶ on the other hand, concluded from his observations that this alkaloid possesses a protective power too slight to be of importance. Auer's results would have been more striking had he employed an intoxicating dose which was one hundred per cent fatal. Mita's experiments might have led to a different conclusion if he had increased the amount of atropine injected. The discordance between the results of these two authors is puzzling.

EXPERIMENT 19. (XI-13-11.) .2 cubic centimeter of one per cent atropine sulphate in physiological salt solution was added to the poison solution just before injection, or else was injected in the same vein at the stated interval before the injection of the poison.

Pig.	Weight.	Atropine Sulphate (grams).	Interval after Atropine.	Quantity (ml. T.).	NaCl. (cc.).	Relation to Body Weight.	Symptoms.	Autopsy.
119.....	190	0.002 intravenously.	O	0.0158	1.58	1 : 12000	xxx D. 2' 30"	Typical.
120.	190	"	O	0.0158	1.58	1 : 12000	O XI-14 R.	—
121.....	185	"	O	0.0123	1.23	1 : 15000	x " R.	—
122.....	180	"	O	0.012	1.2	1 : 15000	x " R.	—
123.....	180	"	30"	0.015	1.5	1 : 12000	O " R.	—
124.....	180	"	30"	0.015	1.5	1 : 12000	O " R.	—
125.....	175	"	30"	0.0118	1.18	1 : 15000	O " R.	—
126.....	205	"	15"	0.0136	1.36	1 : 15000	xxx D. 3' 30"	Typical.

O R = No anaphylactic symptoms — recovery.

Some of the animals showed slight dyspnea, with convulsions and prostration but, with the exception of two, the manifestations were fleeting and recovery took place within ten to thirty minutes. Atropine, therefore, was effective in protecting six out of eight animals (seventy-five per cent) from a lethal or even supra-lethal dose of the poison. The anomalous behavior of Figs 119 and 126 is difficult to explain, since the other six animals consistently failed to develop the characteristic symptoms of the poisonous shock.

2. The protective effect of morphine sulphate:

A similar experiment was carried out with morphine sulphate as it was felt that such a series might be of interest as affording a comparison with the action of atropine. No attempt is made here to analyze the physiological mechanism involved. It is interesting to note the extremely low susceptibility of the guinea-pig to morphia. Subcutaneous and intraperitoneal injections of .060 gram of morphine sulphate produced at most only a feeble narcosis in the animals.

EXPERIMENT 18. (Intravenous injection of III. T.)

Fig.	Weight.	Morphine Sulphate (grams).	Minutes after Morphine Injection.	Quantity (III. T.).	N/NaCl.	Relation to Body Weight.	Symptoms.
108	175	{ 0.0001 * 0.0009 subcutaneously.	1 hour 3' 30"	0.0113	1.13	1 : 15000	O R.
107	177	{ 0.00025 * 0.00075 "	38' 15"	0.0118	1.18	1 : 15000	xxx D. 18' 15"
106	190	0.0005 "	58' 0"	0.0158	1.58	1 : 12000	xxx D. 12'
105	190	0.0010 "	34' 30"	0.0158	1.58	1 : 12000	x D. 6-18 hours.
104	170	0.001375 "	60' 0"	0.0142	1.42	1 : 12000	x D. over 6 hours.
103	170	0.00275 "	50' 0"	0.0142	1.42	1 : 12000	O D. 47'
102	180	0.0055 "	28' 0"	0.015	1.50	1 : 12000	O D. 56'
109	175	0.0025 intraperitoneally.	31' 0"	0.0117	1.17	1 : 15000	O D. 42' 30"
110	185	0.0050 "	35' 0"	0.0123	1.23	1 : 15000	x R.
111	185	0.0075 "	44' 0"	0.0123	1.23	1 : 15000	x D. 66'
112	190	0.010 "	54' 15"	0.0127	1.27	1 : 15000	xx D. 49' 30"
139	220	0.005 intravenously.	0	0.01467	1.47	1 : 15000	xxx D. 3'

140	225	0.010	"	O	0.015	1.5	1 : 15000	x D. 1 hour 28'
141	240	0.010	"	O	0.020	2.0	1 : 12000	O D. 53' 15"
113	185	0.005 intraperitoneally.		O R.
114	190	0.010	"	O R.
115	220	0.020	"	O R.
116	220	0.040	"	O R.
117	225	0.060	"	O R.

* Two injections fifty-four minutes apart.

Of the animals tested only three showed typical symptoms and with two of these death was slightly delayed. The three autopsies revealed typical inflation of the lungs, with epicardial hemorrhages in two. Nine of the pigs had only slight symptoms, and although the issue was fatal, death was delayed from forty-two minutes to over six hours. On section, however, six of the animals showed inflated lungs with epicardial hemorrhages. Two animals recovered. It will be noted that in five cases a dose of 1 : 12000 failed to produce the typical immediate symptoms. Further investigation of the effects of morphia might lead to a better knowledge of the factors concerned in the sequelæ of parenteral protein administration.

3. The protective effect of chloral hydrate :

Of the observations on the effects of chloral upon anaphylactic intoxication those of Banzhaf and Steinhardt may be quoted as having a more pertinent application to the present experiments, since they studied the effects of this drug upon animals treated with a poison obtained by Vaughan's method from egg white. They conclude : "Normal guinea-pigs under the influence of chloral (by intracardiac and intramuscular injections) were completely protected against one and one-fourth fatal doses of the poison (given intracardiacally). If two or more fatal doses were given, death resulted. Chloral mixed with the poison and then given caused irregular results which were interpreted as meaning that there is no chemical union of the chloral and poison in vitro. We assume that the chloral protected by union certain vital cells." The present technic differs from theirs only in the method of administration and the dose of the chloral. The poison, as usual, was injected intravenously instead of intracardiacally. The chloral hydrate was used in a freshly prepared two and one-half per cent solution in salt solution.

EXPERIMENT 20. In the intravenous injections the chloral solution was added to the desired quantity of poison solution immediately preceding the injection. The hypnotic effects of chloral are complete and practically instantaneous when administered by the venous route.

Fig.	Weight.	Chloral Hydrate.	Minutes After Chloral Injection.	Toxophore Injection.		Relation to Body Weight.	Symptoms.
				Quantity.	N/NaCl.		
130	205	0.015 intravenously.	0	0.0137	1.37	1:15000	O R.
132	155	"	0	0.0103	1.03	1:15000	O D. 18 hours.
135	215	"	0	0.0143	1.43	1:15000	O R.
143	210	"	0	0.014	1.40	1:15000	x R.
134	215	"	0	0.0165	1.65	1:13190	O R.
131	215	"	0	0.0179	1.79	1:12000	x R.
142	205	"	0	0.0171	1.71	1:12000	xxx D. 4' 30"
133	195	"	0	0.0215	2.15	1:9074	xxx D. 6' 30"
129	200	"	0	0.0133	1.33	1:15000	O D. 2 hours.
128	195	"	0	0.0162	1.62	1:12000	O D. 2 hours.
127	170	"	0	0.0141	1.41	1:12000	O D. 31' 30"
137	255	0.030 intraperitoneally.	38	0.0166	1.66	1:15000	O R.
136	230	"	19	0.0153	1.53	1:15000	O D. 5 hours.
138	220	0.060 intravenously.	—	—	—	—	Control, O R.

With the exception of two animals displaying typical symptoms, both of which received amounts of poison considerably in excess of that required to kill, six of the thirteen survived the injection with slight or no symptoms, while five succumbed in from two to thirteen hours without exhibiting the classic respiratory spasms. Autopsy showed the typical picture in Figs 133 and 142, while in 127 and 128 there was a partial inflation of the lungs with punctate hemorrhages beneath the pericardium. The results, although not so strikingly positive as those of Banzhaf and Steinhardt, at least tend to confirm their conclusions.

4. The protective effect of lecithin :

Banzhaf and Steinhardt found that lecithin given intraperitoneally in doses from two hundred and fifty to five hundred milligrams or more to serum-sensitized guinea-pigs protected them from a second injection of five cubic centimeters of horse serum given twenty hours later. When lecithin was emulsified with the Vaughan cell-poison or given twenty hours before the poison was injected no protection was afforded.

EXPERIMENT 36. Freshly purchased "Lecithin-Merck, from eggs H.P." was purified by solution in boiling absolute ether, filtration, evaporation of filtrate and precipitation with several volumes of acetone (Kahlbaum). The residue was washed with acetone, air dried, dissolved in water-free ether, filtered and evaporated and dried over sulphuric acid in vacuo. The final preparation was faintly acid. A freshly prepared ten per cent emulsion in physiological salt solution was used for the injections.

A. The lecithin emulsion and the proper dose of poison solution were mixed just before injection. Injections were made intravenously.

Date.	Pig.	Weight.	Lecithin (grams).	III. T.	Volume (cc.).	Symptoms.	Autopsy.
I-9-12	244	195	0.250	1:15000	3.15	xx R.	—
"	243	185	0.250	1:15000	3.15	xxx D. 2'	Typical.
I-10-12	248	220	0.250	1:15000	3.15	O D. 24 hours.	Normal.
I-18-12	264	200	0.250	1:15000	3.83	x R.	—
"	265	190	0.250	1:15000	3.76	xxx D. 1' 10"	Typical.
"	266	195	0.250	1:12000	4.12	xxx D. 3' 30"	"
"	267	200	0.250	1:12000	4.16	xxx D. 13' 15"	"

It was found impracticable to give more than .250 gram of lecithin. Control animals survived the injection of such an amount without showing noteworthy symptoms. Of the four animals receiving the minimum fatal dose of the poison two had slight symptoms and recovered, one had no symptoms but died in twenty-four hours, while the fourth reacted typically. The lecithin evidently had an abortive effect. With the larger dose of poison, lecithin was without protective action.

B. The lecithin emulsion was incubated with the poison solution at 37.5° C. for one hour in order to see if this lipoid had any detoxicating action upon the poison.

Date.	Pig.	Weight.	Lecithin.	III. T.	Volume (cc.).	Symptoms.	Autopsy.
I-18-12	271	195	0.250	1:15000	3.80	O R.	—
"	270	170	0.250	1:15000	3.63	O R.	—
"	269	205	0.250	1:12000	4.21	xx D. 10' 10"	Typical.
"	268	175	0.250	1:12000	3.96	xxx D. 2' 10"	"

That the two animals receiving the usual fatal dose of poison recovered without symptoms would seem to show that the poison had been rendered less potent. That the action of the lecithin must be a feeble one is shown by the behavior of the pigs receiving the larger dose of poison. These results differ from those of Banzhaf and Steinhardt. Their injections were made intraperitoneally, the contact between lecithin and poison was one-half instead of one hour (temperature?) and the amount of lecithin was either much smaller (.050 gram) or much larger (.500 gram).

C. In this series a preliminary intravenous injection of lecithin was given, followed at stated intervals by the poison injection in the opposite jugular.

Date.	Fig.	Weight.	Lecithin (grams).	Volume (cc.).	Interval.	III. T.	Volume (cc.).	Symptoms.	Autopsy.
1-10-12	249	225	0.250	2.5	45 min.	1 : 15000	1.50	O D. 24 hours.	—
"	272	200	0.250	2.5	70 "	1 : 15000	1.33	x R.	—
"	273	190	0.250	2.5	70 "	1 : 15000	1.26	xx D. 6' 30"	Typical.
"	275	185	0.250	2.5	22 hrs.	1 : 15000	1.23	xxx D. 3' 20"	"
"	245	190	0.250*	2.5	23 "	1 : 15000	1.27	O R.	—
"	250	230	0.250	2.5	2 hrs. 50 min.	1 : 12000	1.77	O D. 18 hours.	—
"	251	255	0.500	5.0	1 " 45 "	1 : 12000	2.13	xx D. 22'	One lung inflated.
"	274	185	0.250	2.5	70 min.	1 : 12000	1.54	xxx D. 4'	Typical.
"	246	235	0.250	2.5	22 hrs.	1 : 12000	1.79	x R.	—

* Fig 245 received .050 gram intravenously and .200 gram intraperitoneally.

The primary lecithin injection was without appreciable effect upon the animals. The results are too irregular and inconsistent to warrant conclusions, but from them it is evident that in some cases lecithin exerts a certain prophylactic action against the poison.

The action of lecithin deserves further study. Banzhaf and Steinhardt believe that in the hypersensitive animal this phosphatide absorbs the specific protein-splitting antibody, thus preventing the cleavage and liberation of the poison, and, reasoning further from their results, they conclude that since the lecithin acts upon the ferment and not upon the poison, it therefore is unable to protect the normal animal from the artificially prepared poison. Whether the lecithin acts by a chemical detoxication or by shielding the tissue cells, either by a vital union with them or with a specific ferment, is a question which is beyond the scope of the present communication.

A closer scrutiny of some of the experimental effects brought out by the present study tempts one to speculate regarding their possible significance. The localization of the poisonous reaction offers an inviting field, and certain of the data recorded above are suggestive in this direction. The manifestations produced by a sub-lethal dose of the poison differ essentially from those following the injection of a fatal dose. The respiratory disturbance is less marked and is transitory, while the paralytic symptoms become more evident. It is possible that the velocity of the fatal reaction is so great that the secondary effects are either masked or are never reached. The fact that certain drugs and other agents may prevent the acute respiratory paroxysms and yet fail to save the animal from a slower death may possess a possible meaning which it may not be profitless to heed. The question whether the intoxication in anaphylaxis is peripheral or central may be said to be still open to debate. The effects provoked by the parenteral administration of the artificially obtained, poisonous substance in non-fatal doses, and as modified by atropine, morphine, chloral, and other drugs, seem to suggest the plurality of its action. It is conceivable that the poisonous fraction

obtained by Vaughan's method contains either an essential component which is several in its physiological action, and which in sufficient doses exerts its primary and dominating effect on the respiratory mechanism, or that it contains groups or individual constituents of different selective vital affinities, the most eminent of which is for the peripheral or central cells functioning in respiration. It is not unreasonable to hope that a further separation of the poisonous fraction into its components and a more intimate study of their various actions on the animal economy may furnish valuable clues not only to the relation of these chemical substances to true anaphylactic processes, but also to the physiological nature of these varied phenomena or hypersensitiveness.

V. SUMMARY.

1. Tuberculo-protein treated by the method of Vaughan yields a poisonous substance (called Toxophore by Vaughan), which, in suitable doses, produces in normal guinea-pigs an intoxication resembling, if not identical with, the specific immediate protein intoxication in hypersensitive pigs. The gross pathological findings appear to be similar in both conditions. The minimum fatal dose of two preparations was one part to fifteen thousand parts of body weight.
2. Boiling for one minute with filtration does not affect the potency of the watery solution.
3. Fatal doses of the poison cause an abrupt fall in temperature. Smaller amounts (up to .010 gram for pigs from two hundred to two hundred and fifty grams) are apparently without appreciable effect on the body temperature.
4. Under the experimental conditions noted, repeated increasing doses of poison fail to render animals immune to a minimum fatal dose.
5. Survival from a large intravenous dose of the poison apparently renders the animal refractory, for forty-eight hours at least, to an amount in excess of that required to kill.
6. Fresh brain, lung, and liver tissue, under the conditions noted, showed no binding or neutralizing affinity for the poison.

7. Normal guinea-pig serum has little if any destructive action on the cell-poison.

8. Intradermic injections of .00005 gram of the poison produced no local reactions in normal or sensitized pigs.

9. Atropine sulphate protected seventy-five per cent of the animals from a synchronous injection of fatal amounts of poison.

10. Morphine sulphate aborts the acute symptoms and delays death, and in two cases completely protected from fatal intoxication.

11. Chloral hydrate protected many of the animals against an otherwise fatal dose of the poison, and inhibited the acute manifestations and delayed death in others.

12. Lecithin emulsion injected simultaneously with the poison seems to possess a slight and irregular prophylactic action. Incubation of the poison with lecithin emulsion for one hour at 37.5° C. increases this neutralizing property. A dose of one to twelve thousand of the poison was not affected. The preliminary administration of lecithin protected some of the animals, delayed death in others and was without effect in the remainder. The results were too inconstant to warrant definite conclusions.

[The preparation of the cleavage products and some of the initial experiments were carried out in 1910-11 at the Saranac Laboratory for the Study of Tuberculosis. The authors desire to express their sincere appreciation of the generous courtesy extended by Dr. E. L. Trudeau and of the valuable aid and direction of Dr. Edward R. Baldwin. To Dr. Allen K. Krause and Mr. Victor Hugo are due thanks for much helpful assistance. This part of the work was made possible through the generosity of Mrs. A. A. Anderson of New York City. The major part of the animal experiments was done at the Hoagland Laboratory, Brooklyn.]

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HAROLD C. ERNST, M.D.,
Editor of The Journal of Medical Research,
240 Longwood Avenue,
Boston, Massachusetts, U.S.A.

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THE ANTIBODIES PRODUCED BY VARIOUS CONSTITUENTS OF DOG'S BILE.*

HOWARD T. KARSNER, M.D., BOSTON.

AND

RICHARD M. PEARCE, M.D., PHILADELPHIA.

*(From the Laboratory of Pathology (Phillips Fund) Harvard Medical School,
and the John Herr Musser Department of Research Medicine, University
of Pennsylvania.)*

In a previous publication by one of us¹ it was shown that the injection of dog's bile into rabbits produced a serum which was non-hemolytic but strongly agglutinative for dog's red corpuscles. This fact has been demonstrated also by Ruffer and Crendiropoulo² in the case of ox bile and ox corpuscles. In some of the anti-sera thus produced a moderate hemolytic power has been developed, but in all cases the hemagglutinative property of the serum has been far more striking. Ruffer and Crendiropoulo explained the phenomenon by demonstrating an anti-hemolytic property on the part of the serum, using bile as the hemolysin. The objection to the use of bile as a hemolysin lies in the fact that its hemolytic activity depends on the chemical action of its salts and is quite apart from the immuno-chemical properties of such a hemolysin as can be found in an immunized animal, *i.e.*, a hemolytic amboceptor whose action must be completed by the presence of complement.

It has occurred to us that it might be profitable to learn whether or not there is some chemical fraction of bile which

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might be responsible for the peculiarity of anti-bile serum, *i.e.*, to learn whether or not some ingredient, perhaps some product of blood destruction, leads to the production of an agglutinin, an ingredient not present in other body fluids, such as urine and serum, which fluids although producing agglutinins also produce a much more powerful hemolysin.³

It was determined, therefore, to fractionate dog's bile and determine in vitro the hemolytic, anti-hemolytic, agglutinative and anti-agglutinative powers of the fractions. It was determined also to immunize rabbits to the various fractions and to determine in the same way the properties of the immune sera so produced. The study has led into certain problems of hemagglutination which have thrown additional light upon the results. These are taken up in the discussion which follows the statement of the results of our experiments.

We present, in sequence, the technic employed, the results of the experiments, the discussion of results with a few additional and explanatory experiments and conclude with a summary of the work.

TECHNIC.

A. The preparation of the bile fractions (in the preparation no heat was used except that in making the fractions used for immunizing one series of rabbits the evaporation of the alcohols was aided by gentle heat over the water bath. The immune sera prepared with these fractions were quite as active as those prepared with the non-heated fractions):

Fraction I. represents chiefly bile mucin plus a small amount of pigment. It is the precipitate thrown down by the addition to one hundred cubic centimeters bile, after acidifying with acetic acid, of ten volumes of ninety-five per cent alcohol. It was collected on a filter and made up to one hundred cubic centimeters with .85 per cent salt solution, complete solution being aided by a few drops of saturated sodium hydrate solution. According to Hoppe-Seyler mucin equals 3.49 parts in one thousand of dog's bile.

Fraction II. represents probably certain protein and other organic constituents aside from mucin; possibly also some inorganic salts thrown out of solution in the process. The filtrate, after the separation of Fraction I., was evaporated to a gum and one liter of absolute alcohol added. The insoluble portion was collected and made up to one hundred cubic centimeters with .85 per cent salt solution. According to Hoppe-Seyler this fraction is equal to six parts in one thousand of dog's bile.

Fraction III. represents the bile salts (Plattner's crystallized bile). The filtrate after the separation of Fraction II. was evaporated to small bulk and precipitated by the gradual addition of one liter of ether. It was collected and made up to one hundred cubic centimeters with .85 per cent salt solution. According to Hoppe-Seyler it equals 122.8 parts in one thousand of dog's bile.

Fraction IV. A represents the fats and lipoids. The filtrate after the separation of Fraction III. was evaporated to small bulk and purified by reprecipitation with ether three times. It was then evaporated to small bulk and made up to one hundred cubic centimeters with .85 per cent salt solution. According to Hoppe-Seyler this fraction equals: fats, 15.11 parts; soaps, 16.03 parts; lecithin, 18.11 parts, and cholesterin, 2.97 parts per one thousand of dog's bile.

Fraction IV. B. — In the preparation of Fraction IV. A, a thick, oily or gummy substance was noticed settling out of the ether solution. This was separated and found to be insoluble in ether, but soluble in alcohol and water. Its exact composition is not known, although it appears to be made up chiefly of soaps. Prepared from one hundred cubic centimeters bile, two hundred cubic centimeters salt solution were used to dissolve it.

B. The immunization of the rabbits :

All injections were made at five-day intervals except in the case of the animals immunized to whole bile; in the latter animals an injection was never given until the animal had recovered the weight lost following the preceding injection. The first injection was of three cubic centimeters whole bile subcutaneously, the second of four cubic centimeters subcutaneously, and the succeeding three of five cubic centimeters intraperitoneally, each dose being diluted with fifty cubic centimeters .85 per cent salt solution. The fractions were given in the same amounts and in the same way, except that they were not further diluted with salt solution. The animals were bled from the femoral artery one week after the last injection.

C. The titration of antigens and immune sera :

By titration is meant the determination of the hemolytic, agglutinative, anti-hemolytic, and anti-agglutinative properties of the sera and their antigens.

Hemolysis and agglutination were determined in the same test-tubes. For each antigen or anti-serum four rows of tubes were used, one for the determination with washed blood, the next for the determination with washed blood plus .1 cubic centimeter guinea-pig complement, the third with washed blood plus .1 cubic centimeter dog complement. In the fourth the action of the agent on unwashed dog's blood was determined. Into one tube of each series was placed .5 cubic centimeter of the agent under investigation (antigen or anti-serum), into the next .5 cubic centimeter of a 1/2 dilution, into the third .5 cubic centimeter 1/4 dilution,

into the next .5 cubic centimeter 1/8 dilution, and so on up to 1/256. To each tube was added .5 cubic centimeter ten per cent suspension of dog's blood, in three rows washed, and in the fourth unwashed. This made the original dilutions of serum range between 1/2 up to 1/512 because of doubling the volume in the tubes. Each tube of the second row received .1 cubic centimeter fresh guinea-pig serum and each of the third row .1 cubic centimeter fresh dog serum. The tubes were kept at room temperature and readings made at one-half, one, two, and four hours for the determination of both agglutination and hemolysis.

For the determination of anti-hemolytic properties, the serum of a rabbit immunized to whole dog's blood was employed as a hemolysin. Guinea-pig complement was used throughout. The agents to be tested were diluted as in the preceding series and placed in two rows of tubes in amounts of .5 cubic centimeter. To each tube of the first row was placed .5 cubic centimeter of a mixture of washed dog's blood, one dose of guinea-pig complement, and two doses of hemolysin. In the second row was placed the same mixture except that there was only one dose of hemolysin. The variation in dose of hemolysin was arranged in this fashion so as to determine whether any anti-hemolytic action that might be present was weak or strong. If it appeared in the first row, it must be fairly strong to oppose the double dose of hemolysin, if in the second row, it would be correspondingly weaker. In this series the hemolysin was titrated in the incubator at 37° C. for one hour and the tests for anti-hemolysin performed under the same conditions.

In order to determine the presence of anti-agglutinins the same immune serum as in the preceding series was used as an agglutinin. The same system of dilutions was employed and .5 cubic centimeter placed in each of two rows of tubes. In one row was placed .5 cubic centimeter of a mixture of washed dog's blood and one minimal dose of agglutinin, in the other row the same with a double dose of agglutinin. The tests were performed at room temperature and readings made every one-half, one, two, and four hours.

As an example of the technic employed we append a complete protocol of Fraction I. and its immune serum (see Charts I. and II.).

RESULTS OF TESTS.

Fraction I. — This fraction showed no hemolytic or agglutinating action on washed blood, washed blood with dog and guinea-pig complement or on unwashed blood. It had a moderate anti-hemolytic action, in dilutions as high as one-sixty-fourth hindering the hemolytic action of one dose of hemolysin, and in dilutions of one-sixteenth hindering the action of two doses of hemolysin. It showed no anti-agglutinating power whatever.

Of four sera prepared at different times, two were distinctly agglutinative, one somewhat less so, and one not at all so. All four were very slightly hemolytic except in the presence of guinea-pig complement, under which conditions hemolysis appeared in dilutions of one-eighth at the end of one-half hour, at room temperature, and one-sixteenth at the end of one hour. Of the four sera, two were tested for anti-hemolytic power and one showed distinct limitation of hemolysis with both a single and double dose of hemolysin. Where anti-hemolytic power was seen, agglutination was prominent. This serum favored rather than hindered agglutination. The second serum showed the same properties to a somewhat limited degree.

Fraction II. — This fraction showed neither hemolytic nor hemagglutinative properties. It very slightly hindered the action of one dose of hemolysin in as high a dilution as $1/512$ and hindered a double dose of hemolysin in a dose of one-half. It also showed a very slight anti-agglutinating power in dilutions of one-half and one-fourth.

Three immune sera produced by this fraction were studied. Of these one was inactive as a hemolytic or agglutinative agent, and one showed very moderate agglutinative power, — dilutions of one-fourth with dog and guinea-pig complement. This latter serum as well as the third serum showed slight hemolytic power only in the presence of guinea-pig complement. Two of these sera were studied for anti-agglutinative and anti-hemolytic properties; the less active agglutinator showing a slightly higher anti-hemolytic power, present against one dose of ambóceptor in dilutions of one-eighth. In these tubes, however, there was considerable agglutination. These sera favored rather than hindered agglutination.

Fraction III. — This fraction was distinctly hemolytic, but not at all agglutinative. The hemolytic power was evident in dilutions of one-sixteenth at the end of one-half hour and in dilutions of one-thirty-second at the end of one hour. Dog complement and guinea-pig complement did not influence it. In low dilutions ($1/16$) it favored hemolysis by the

specific serum, but in higher dilutions ($1/32$ to $1/128$) it hindered the action of two doses of hemolysin and in still higher dilutions ($1/32$ to $1/512$) hindered the action of one dose of hemolysin. It did not hinder agglutination except in so far as it was hemolytic.

Two immune sera were studied, both of which showed slight agglutinative activity, one in dilutions of one-half, the other in dilutions of one-fourth. Neither serum showed hemolytic activity. One of the sera was tested for anti-hemolytic activity and produced slight interference with the action of one dose of hemolysin in dilutions as high as $1/512$. It did not interfere with two doses of hemolysin in any dilution. This serum showed no anti-agglutinative action.

Fraction IV. A. — In low dilutions ($1/2$, $1/4$) this fraction colored the blood suspensions a dark brown and produced a yellowish brown precipitate in the bottom of the tubes. In higher dilutions a less marked discoloration appeared and hemolysis was noted after from one to two hours. The precipitation probably is due to the soap content of this fraction, inasmuch as it closely resembles the action of saponin and of sodium oleate. The same precipitation prevented hemolysis in the series of experiments for the determination of anti-hemolytic activity, but in dilutions above one-eighth where no precipitation occurred, hemolysis was unhampered. There was no interference with agglutination except by the same process of precipitation in low dilutions.

Two immune sera were prepared, neither of which was hemolytic, agglutinative, anti-hemolytic or anti-agglutinative.

Fraction IV. B. — This showed slight hemolytic action, producing partial hemolysis in dilutions of one-half and one-fourth. It showed no agglutinative action whatever. It was neither anti-hemolytic nor anti-agglutinative.

Two immune sera were prepared which were not agglutinative, hemolytic, anti-hemolytic or anti-agglutinative.

Whole bile showed hemolytic activity in dilutions as high as one-thirty-second. There was no agglutination. It hindered hemolysis by one dose of hemolysin in dilutions of $1/8$

to $1/512$ and by two doses of hemolysin in dilutions of $1/8$ to $1/256$. The lower dilutions ($1/2$, $1/4$) showed hemolysis by the bile itself. It was anti-agglutinative only in so far as the addition of bile in low dilutions produced hemolysis.

Three immune sera were studied, all of which were both agglutinative and hemolytic, but showed agglutination in higher dilutions than those in which hemolysis appeared. With all three sera hemolysis was greater in the presence of guinea-pig complement than under other conditions. It was somewhat less marked when no complement was used, next in the presence of a large dose of dog complement, and least marked when used with unwashed dog's blood. Agglutination was most marked in the presence of a large dose of dog complement and with unwashed dog blood and appeared to the same degree in both series. It will be noted, however, that whereas agglutination was the same in both, hemolysis was more marked with the larger dose of dog complement. It was possible to test only one serum for anti-hemolytic and anti-agglutinative power, neither being demonstrated.

In the earlier experiments of Pearce,¹ dilutions of one-tenth and one-twentieth were used to determine the activity of the serum on unwashed fresh dog blood, thus approximating conditions *in vivo*. No complement was added. In the present series, however, stronger dilutions were employed and it was found that the dilutions one-half, one-fourth, one-eighth showed hemolysis. This accounts for the slight variation from the earlier conclusions.

Controls. — A considerable number of normal rabbit sera were used as controls. Occasionally a serum was found which was slightly agglutinative in dilutions of one-half and one-fourth and in such a serum traces of hemolysis appeared in the same dilutions. At no time did any normal serum show anti-agglutinative or anti-hemolytic activity.

DISCUSSION OF RESULTS.

In order to facilitate comparison and to simplify the results of the tests, a chart is appended (see Chart III.) which tabulates a summary of the various immunological

properties shown by the fractions and by their corresponding immune sera. It must be understood, however, that this chart represents what are considered to be type reactions and that the reader should refer to the "Results of Tests" in order to examine the results in detail.

It can readily be seen that the fractions most active in the production of immune sera were Fractions I. and II. These fractions contain practically the entire protein constituents of bile and, since the production of demonstrable immunity depends almost exclusively on the introduction into the organism of protein bodies, the result obtained corresponds with what might have been predicted. Of these two fractions, however, Fraction I., made up chiefly of mucin, produced the immune serum most strikingly similar to an anti-bile serum in that it showed slight hemolytic and well-marked hemagglutinative properties as well as a well defined anti-hemolytic power. A comparison of two such sera with two sera produced by the injection of whole bile shows the serum produced by Fraction I. to be quite as active an agglutinator as that produced by injecting whole bile; the hemolytic action of the former, however, is even less than that of the latter. As contrasted with the serum produced by Fraction I., that produced by Fraction II. showed no such special properties. It was moderately hemolytic and only slightly agglutinative, properties which usually appear after the injection of other body fluids such as urine and blood serum.^{1,2} Its agglutinative power was not greater than occasionally appears in normal rabbit serum. The greater activity of the immune sera of Fraction I. as contrasted with that of Fraction II. is not the result of greater protein content of Fraction I. because, according to Hoppe-Seyler, the protein content of Fraction II. is a third again as much as that of Fraction I. The high agglutinative activity of the immune sera of Fraction I. must be looked upon as a special character of sera prepared by the injection of this fraction. It is of interest to know that among about thirty normal guinea-pig sera used at various times for complement in the series of tests, one showed well-marked agglutinative

action on dog's corpuscles; indeed, one entire day's work had to be ruled out because of this fact. It was found that, although inactive in other respects, the immune sera produced by Fraction III. showed slight agglutinative power; but again this cannot be considered an artificially induced property of these sera because it does not exceed that of some normal sera. It is quite apparent that Fractions IV. A and IV. B. produced no active immune sera. The results with the immune sera of Fractions III., IV. A, and IV. B are quite consistent with the findings of immunologists generally, that the injection of salts, fats, lipoids, and soaps does not produce immune sera. It would seem fair to conclude, therefore, that the most important special properties of immune sera prepared by the injection of dog's bile depend rather upon the mucin and other protein content of that fraction described in this paper as Fraction I. than upon any other constituent of whole bile. The immune serum of Fraction I. also exhibited well-marked anti-hemolytic power (see Chart II. D), but it was noted at the time that all the tubes in which this was evident showed considerable agglutination. In spite of this it was thought that the anti-hemolytic power might depend upon an anti-complementary property of the immune serum. This was tested for with a sheep system, as shown in Chart IV., but could not be definitely demonstrated when the final readings were made. The complements and sera were so carefully titrated that only the end result can be accepted, yet it must be stated that hemolysis with the single dose of dog complement proceeded somewhat more slowly than the control. This could be interpreted as indicating a very slight anti-complementary property on the part of this particular serum. Such a property could not be ascribed to the age of the serum because the tests were applied within a week of the time of withdrawal, the sera being very effectively refrigerated in the meantime. That it is sufficient to cause the result shown in Chart II. D, however, is hardly to be considered, this being especially so when the fact is remembered that guinea-pig complement was used for the series reported in Chart II. D.

The well marked agglutinating power of the immune serum of Fraction I. made it impossible to determine whether or not there was some special property capable of acting on the dog amboceptor or dog corpuscles to hinder hemolysis. There was left therefore only the possibility that the rapid agglutination of the corpuscles hindered their solution. The only argument against this is the fact that at the same time agglutination is taking place, sensitization with the specific amboceptor also is occurring and ultimately this should lead to more ready solution of the corpuscles. To put the matter to the test the following experiment was instituted, as protocolled in Chart V. Two rows of test-tubes were used; into both rows were placed .5 cubic centimeter five per cent suspension of washed dog's blood; into one row was placed in addition .5 cubic centimeter of dilutions of specific anti-dog corpuscle immune serum in the proportions indicated. All the tubes and the remaining immune serum to be added later to the second row were incubated one-half hour, at the end of which time the row containing immune serum showed well marked agglutination throughout, less marked, however, in the high than in the low dilutions. Amounts of immune serum equivalent to those placed in the first row were added to the second row of unagglutinated corpuscles, and .1 cubic centimeter fresh guinea-pig complement added to all the tubes. These were then incubated for one hour, readings being taken at one-fourth, one-half, and one hour; the tubes stood at room temperature for seventeen hours additional and a final reading was made. The results show that agglutination by a specific serum, in spite of the fact that sensitization of the corpuscles occurs simultaneously, very distinctly hinders hemolysis; and that the more marked the agglutination, as seen in low dilutions, the more noticeable is the hindrance to hemolysis. We do not wish to imply that this explanation is the only one to be offered for the anti-hemolytic action of the immune serum of Fraction I., but there can be no doubt that it plays an important part in the phenomenon.

In comparing the results obtained by the use of the

immune sera with those obtained by the use of the fractions themselves no other conclusion can be drawn than that no definite relation is to be demonstrated. It might seem that the fact that Fraction III. is hemolytic explains the fact that its immune serum is not hemolytic, but in order to be convinced that there is any relation between the two facts we should be able to demonstrate an anti-hemolytic property in the serum. This could not be done. Furthermore, the dependence of the hemolysis on the toxic salt content⁴ and concentration of this fraction removes the ground for the belief that its immune serum should be anti-hemolytic.

That this does not completely settle the matter is shown by a critical examination of Chart II., where it will be seen that, under the various conditions imposed, the immune serum behaved differently. Thus the most marked hemolysis appeared in the presence of guinea-pig complement, next most markedly with unwashed dog's blood, next with a fairly large amount of dog complement and least with that proportion of dog complement found in unwashed blood. There is but one explanation for this and that is to conclude that dog serum in proper doses has some intrinsic anti-hemolytic power in the presence of its specific immune serum. That this is not limited to an immune serum prepared by the injection of bile is shown by the fact that the same relative findings were demonstrated with the use of an immune serum prepared by the injection of dog blood. In both cases this property resides in some agent which is destroyed by heating the dog serum to 56° C. for one-half hour. It appears to reach its maximum in doses of dog serum approximating the proportions found in unwashed dog's blood, because it is less marked as larger amounts of fresh homologous dog serum are added. The latter phenomenon may depend to a certain extent upon the well-known fragility of dog erythrocytes. In experiments with hypotonic salt solutions we have found hemolysis to be complete in .35 per cent NaCl solution and partial as high as .60 per cent NaCl solution. No experiments have been tried with smaller amounts of dog serum than are found in unwashed dog blood; nor is

this property confined to dog blood, for Muir and Browning,⁵ working with the effect of an ox serum on its homologous hemolytic system, have attained somewhat similar results, dependent, they think, on an agglutinative property of fresh ox serum under the conditions of the experiment. We have been unable to confirm Muir and Browning's results in the latter respect, although applying their technic in detail to the sera at our command. Indeed, our results would indicate that the fresh dog serum in proper doses exercises a slight anti-agglutinative as well as anti-hemolytic power. For evidence of this, note the fact that in Chart II. B agglutination is less marked with unwashed dog blood than in the presence of greater amounts of dog complement. Further, the use of dog complement does not produce agglutination sooner or in higher dilutions than with the employment of guinea-pig complement, as should be the case were we to assign a specific pro-agglutinative action to fresh dog serum. Hence, although the anti-hemolytic property of the immune serum of Fraction II. may be and almost certainly is due to its agglutinating power, the anti-hemolytic power of dog serum is something more specific. The exact degree of specificity cannot be taken up at the present time.

Final Dilutions of tion, ½ cc. to each	1/512			NaCl.			
	1 Hour.	2 Hours.	4 Hours.	½ Hour.	1 Hour.	2 Hours.	4 Hours.
Observation Time .							
Washed dog blood, pension	-	-	-	-	-	-	-
Washed dog blood (1 cc. fresh guinea-pig	-	-	-	-	-	-	-
Washed dog blood (1 cc. fresh dog serum	-	-	-	-	-	-	-
Unwashed dog blood, pension	-	-	-	-	-	-	-
Washed dog blood (1 cc. fresh guinea-pig	-	-	-	-	-	-	-
Washed dog blood (1 cc. fresh dog serum	-	-	-	-	-	-	-
Unwashed dog blood	-	-	-	-	-	-	-
Each tube contains 8 of anti-dog's blood and 10% suspensio blood + equal part tion	+	+	+	+	+	+	+
As above except tha tains 1 minimal do	-	-	+	-	-	-	+
Each tube contains sin + 1 dose comp pig) + proper blood suspension fraction dilution	C				C		
As above except tha tains 1 dose hemo	C				C		

Final Dilutions of Serum, $\frac{1}{2}$ each Tube	$1/512$				NaCl.			
	4 Hours.	$\frac{1}{2}$ Hour.	1 Hour.	2 Hours.	4 Hours.	$\frac{1}{2}$ Hour.	1 Hour.	2 Hours.
Observation Time	4 Hours.	$\frac{1}{2}$ Hour.	1 Hour.	2 Hours.	4 Hours.	$\frac{1}{2}$ Hour.	1 Hour.	2 Hours.
Washed dog blood, $\frac{1}{2}$ cc. 10 pension	-	-	-	-	-	-	-	-
Washed dog blood (as above cc. fresh guinea-pig serum	-	-	-	-	-	-	-	-
Washed dog blood (as above cc. fresh dog serum	-	-	-	-	-	-	-	F
Unwashed dog blood, $\frac{1}{2}$ cc. 10 pension	-	-	-	-	-	-	-	-

Washed dog blood (as above)	-	-	-	-	-	-	-	-
Washed dog blood (as above cc. fresh guinea-pig serum	-	-	-	-	-	-	-	-
Washed dog blood (as above cc. dog serum	-	-	-	-	-	-	-	-
Unwashed dog blood (as above)	-	-	-	-	-	-	-	-

Each tube contains 8 minimal of anti-dog's blood immune and 10% suspension washed blood + equal parts diluted immune serum, Fraction I.	+	+	+	+	+	+	+	+
As above, except that each tube contains 1 minimal dose of anti blood immune serum	+	-	-	-	+	-	-	+

Each tube contains 2 doses of hemolysin + 1 dose guinea-pig immune serum + proper amount dog blood corpuscles suspended Equal parts of dilutions immune serum added, Fraction I.	P	C
As above except that each tube contains 1 dose hemolysin	P	C

CHART III.

REFERENCE TABLE OF PROPERTIES OF VARIOUS ANTIGENS AND IMMUNE SERA.

	Hemolysis.	Agglutination.	Anti-hemolytic Power. (1/64)	Anti-agglutinative Power.		Hemolysis.	Agglutination.	Anti-hemolytic Power.	Anti-agglutinative Power.
Fraction I.	None.	None.	Moderate. (1/64)	None.	Immune sera, Fraction I.	Slight.	Well marked.	Well marked.	None.
Fraction II.	None.	None.	Slight.	Slight.	Immune sera, Fraction II.	Moderate in one of three.	Very slight.	Slight.	None.
Fraction III.	Marked.	None.	Moderate.	None.	Immune sera, Fraction III.	None.	Very slight.	None.	None.
Fraction IV. A . . .	Slight.	None.	None.	None.	Immune sera, Fraction IV. A . . .	None.	None.	None.	None.
Fraction IV. B . . .	Slight.	None.	None.	None.	Immune sera, Fraction IV. B . . .	None.	None.	None.	None.
Whole bile	Moderate.	None.	Well marked.	None.	Immune sera, whole bile	Moderate.	Well marked.	None (?)	None.

CHART IV.
TABLE OF ANTI-COMPLEMENTARY POWER OF IMMUNE SERUM, FRACTION I.

Guinea-pig complement	1 dose.	2 doses.	3 doses.	1 dose.	—	Dog complement	1 dose.	2 doses.	3 doses.	1 dose.
Immune serum, Fraction I.	0.1 cc.	0.1 cc.	0.1 cc.	—	1 cc.	Immune serum, Fraction I.	0.1 cc.	0.1 cc.	0.1 cc.	—
Anti-sheep's blood, immune serum	2 doses.	2 doses.	2 doses.	2 doses.	2 doses.	Anti-sheep's blood, immune serum	2 doses.	2 doses.	2 doses.	2 doses.
Sheep's corpuscles (washed—5%)	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	Sheep's corpuscles (washed—5%)	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.
Result	C.H.	C.H.	C.H.	C.H.	—	Result	C.H.	C.H.	C.H.	C.H.

CONCLUSIONS.

1. Immune sera, prepared by the repeated injection of whole dog bile into rabbits, display moderate or slight hemolytic property and well marked agglutinative action on dog erythrocytes. Immune sera, prepared by the repeated injection into rabbits of the various dog bile fractions indicated in this communication, have variable properties, the most important of which follow: The sera of Fraction I. (chiefly mucin) are strongly hemagglutinative and but slightly hemolytic; the sera of Fraction II. (remaining proteins) are slightly hemolytic and not more hemagglutinative than occasional normal rabbit sera; the sera of Fraction III. (bile salts) are not hemolytic and not more agglutinative than occasional normal sera; the sera of Fractions IV. A and IV. B (fats, soaps and lipoids) are neither hemolytic nor hemagglutinative.

2. The greater prominence of the hemagglutinative action of sera prepared by repeated injection of whole dog bile depends upon several factors: (a) The constituents of the bile precipitated by acidifying and the addition of a large bulk of ninety-five per cent alcohol (spoken of as Fraction I. and containing principally mucin) produce, after repeated injection into rabbits, a relatively strong hemagglutinative immune serum. (b) The same serum is only slightly, or not at all, hemolytic because it possesses certain anti-hemolytic powers, in large part, if not wholly, dependent upon its agglutinative properties, certainly not dependent on any clearly demonstrable anti-complementary activity. (c) Immune sera specific for dog blood, and activated by dog complement, are limited in their hemolytic action by the fact that, in certain proportions, dog serum is anti-hemolytic for its specific immune serum.

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A COMPARATIVE STUDY OF ANTIBODIES.*

JOHN A. KOLMER, M.D.

(Professor of Pathology, Philadelphia Polyclinic; Instructor of Experimental Pathology, University of Pennsylvania; Pathologist, Philadelphia Hospital for Contagious Diseases.)

(From the Laboratory of Experimental Pathology, University of Pennsylvania.)

In any given immune serum several different antibodies may be found. Usually a particular one predominates and different antigens stimulate the production of different antibodies. However, a single antigen frequently produces several different antibodies which may be sharply differentiated from one another.

In the minds of not a few persons the various antibodies are poorly differentiated and their properties not well understood. They are frequently regarded as purely protective or curative substances and closely allied one to the other.

A few of the immune bodies are distinctly curative or prophylactic because they neutralize the effects of the antigens or actually destroy them. Such are diphtheria and tetanus antitoxin and a few of the bacteriolytic sera, as anti-meningococcus serum. These are true antibodies in a strict sense of the term. Others, notably the agglutinins, are probably not curative or prophylactic or, in other words, not directly opposed to the antigens, and to these the term "immune body" is better adopted. Antibodies of the latter type may represent reactive substances on the part of the body cells and bear but an indirect relation to the antigen. Thus in different diphtheria immune sera, which are typical examples of the simplest of receptors of the first order, may be found opsonins and a complement fixing body. The serum is not hemolytic or bacteriolytic, yet there is present a body intimately associated with complement and therefore resembling a receptor of the third order.

The various antibodies are best classified, studied, and

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understood according to Ehrlich's theory of their production. Any substance capable of union with the protoplasm of cells may stimulate the production of a corresponding antibody. The antibodies are divided into three main groups according to the structure of the receptors and depending somewhat upon the physical state and structure of the invading substance or antigen. Thus a soluble antigen, as diphtheria toxin, produces the simplest receptor — one composed of a single haptophore group for direct union with the toxin and without a second or zymophore group. The cell can also dispose of the anchored toxin molecule without the aid of a secondary ferment or complement. These are receptors of the first order and it was upon these that Ehrlich first based the principles of his theory. Later other antibodies were found of a more complex nature and by degrees the theory was developed to explain the nature and structure of the new substances. If the invading substance or antigen is an organized body, as a blood corpuscle or an organism, the cell may need more assistance in disposing of them and accordingly these receptors of the second order have in addition to the usual haptophore group a second more specific zymophore group. The agglutinins and precipitins are classified as receptors of this order, the zymophilic group agglutinating or precipitating the anchored molecule to bacterial cell for assimilation or destruction. The opsonins are more difficult to classify but probably belong to this class, the zymophore or opsoniferous group lowering the resistance of the invading microörganism or in some manner preparing it for destruction mainly by the leucocytes of the blood. Receptors of this order do not require the aid of a ferment or complement. For the management and assimilation of still more complex bodies the cell requires the aid of a special ferment. Accordingly we find receptors provided with the usual haptophore group for anchorage to the cell and a second haptophore group to anchor the ferment or complement. The complement really represents the toxophore group of a second degree receptor, only it differs in one particular in being separate from the

receptor until bound by the special haptophore or complementophile group. According to Ehrlich there are present in sera many different complements, which view is directly opposed to that of Bordet and Buchner, who believe in the unity of complement. The special name of "amboceptor" has been applied to receptors of the third order with the belief that they act simply as interbodies between cell and complement and are not "sensitizers" as believed by Bordet. Hence the function of the complements is especially important. If bactericidal amboceptor supplied to cells is capable of combining with hemolytic complements then there may be established a direct relation between the degree of complement fixation and bactericidal activity of the serum. This is still a matter of dispute and the present study bears upon this subject. To this group of receptors belong the lysins: hemo-, bacterio-, cyto- lysins.

In normal serum different kinds of amboceptors may be found, although after immunization the amboceptors differ greatly from those normally found in their cytophile and complementophile groups. In immune serum as in normal serum the various antitoxic, antifermentative, precipitating, agglutinating, hemolytic, and bacteriolytic powers are found to be due to independent and distinct substances. While the properties of these individual bodies are fairly well known, their relation to each other in any given immune serum requires further study. Having on hand a number of fresh immune sera, such a study was undertaken with particular attention to the relation between complements and the various immune bodies present.

Working with streptococcus immune sera I found, with others, that it was possible to a certain degree to differentiate among various strains by complement-fixation tests with homologous antigens. Having found experimentally that there is present in diphtheria immune serum a substance which will fix complement with antigens of diphtheria bacilli I endeavored to ascertain if these complement-fixing bodies were specific for the particular culture used in their production. The results of this study are given under a separate

communication, but as a summary of the work it may be stated here that no specific relation was demonstrable. It was proved, however, that there is present in diphtheria immune serum a complement-binding body specific with diphtheria antigens. Eight different cultures were employed, including virulent and non-virulent diphtheria bacilli and a typical Hofmann's bacillus. Arguing from the behavior of the streptococcus immune serum, we thought that since differentiation among the various cultures was not shown this result may be considered additional evidence of the fact that Hofmann's bacillus belongs strictly to the diphtheria group, but as a result of varied and unfavorable circumstances has become so changed in its chemical and morphological structure as to give it more or less stable characteristics. As would be expected, between this well-marked example of "mutation" and the true diphtheria bacillus may be found strains in partial "mutation," and hence the large group of "pseudo-diphtheria bacilli."

From the fact that diphtheria antitoxin is composed of receptors of the first order it was not expected that any relation would be found between the antitoxic strength of the serum and complement fixation because the ferment is unnecessary, the cell being able to manage the toxin without secondary aid. This is fully shown in the following results.

The purpose of this study was mainly as follows:

1. To study the occurrence and degree of complement fixation with diphtheria immune sera and various antigens, including antigens of diphtheria bacilli. Also to determine if there was any relation between the occurrence and degree of complement fixation and the protective substances in the sera.
2. To study the relations of the different antibodies in various sera with special attention to complement-fixation experiments.

DIPHTHERIA IMMUNE SERUMS.

1. Complement-fixation tests:

(a) Immune serums: Samples of fresh serum were sent me within a few days after bleeding by Dr. A. P. Hitchens, of Glenolden, Pa., Dr. Ranzohoff, of New York City, and by the Dr. Alexander Company of Marietta, Pa. To these gentlemen I wish to express my appreciation of their kindness in sending me the serum and for determining the antitoxin content. No preservatives were added and the reactions were set up the same day the serums were received, the antigens being held in readiness. Serums were inactivated by heating to 55° C. for thirty minutes.

(b) Antigens: These were prepared of the following eight cultures. Sugar acid production and virulence tests were conducted with each culture. The types are recorded after Wesbrook's classification:

TABLE I.
Diphtheria cultures.

No. of Culture.	Source of Culture.	Type of Bacillus.	Sugar Tests.							Inoculation Tests.			Remarks.
			Sacch.	Glucose.	Dextrin.	Mannite.	Levulose.	Lactose.	Inulin.	Hours Grown in Bouillon.	Weight of Pig in Grams.	Dose in cc.	
1 . .	Clin. diph.	B. & C.	—	+	+	—	?	+	—	72	260	1.3	Died.
2 . .	Healthy penis.	C ₁	—	—	—	—	—	?	—	72	282	1.4	Negative.
3 . .	Par k's Bac. No. 8.	Granular.	—	+	+	—	?	+	—	72	274	1.3	Died.
4 . .	Throat conval. diph.	C	—	+	+	—	—	—	—	72	290	1.5	Negative.
5 . .	Otitis media.	C ₂	+	—	—	—	—	?	—	72	300	1.5	Negative.
6 . .	Nasal diph.	D ₂	—	+	—	—	+	+	—	72	284	1.4	Died.
7 . .	Throat carrier case.	C ₃	—	—	—	—	?	+	—	72	260	1.3	Negative.
8 . .	Normal nose.	Hofmann's Bac.	—	—	—	—	—	—	—	72	275	2.5	Negative.

The preparation of the antigens consisted essentially in growing the cultures upon blood serum media for twenty-four hours; washing off the growths with physiological salt solution and centrifugalizing the emulsion; drying the bacilli in a cold place over calcium chloride and sulphuric acid; grinding the bacilli with powdered glass and mixing .5 gram of the dried bacilli with each ten cubic centimeters of .85 per cent NaCl solution containing five per cent of phenol; shaking the emulsion with glass beads for forty-eight hours; centrifugalizing thoroughly; the supernatant fluid was stored away in colored glass bottles in the refrigerator. Complement, antigen, and serum were mixed and the total quantity brought up to five cubic centimeters by the addition of .85 per cent of salt solution. Tubes were incubated at 37° C. for one hour and then two units of hemolysin and one cubic centimeter of the five per cent suspension of corpuscles added. Tubes were incubated for two hours and then placed in the refrigerator for twenty-three hours when results were noted.

The anticomplementary and antigenic powers of the various antigens were then determined as follows:

TABLE 2.
Fixing and anticomplementary power of antigens.

	Amount of Ant., cc.	Ant. No. 1.	Ant. No. 2.	Ant. No. 3.	Ant. No. 4.	Ant. No. 5.	Ant. No. 6.	Ant. No. 7.	Ant. No. 8.
Normal serum, .3 cc.	.04	C.H.*	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
	.06	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
	.08	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
	.1	C.H.	C.H.	C.H.	C.H.	C.H.	S.I.H.	S.I.H.	S.I.H.
	.15	C.H.	C.H.	C.H.	C.H.	S.I.H.	M.I.H.	S.I.H.	M.I.H.
	.18	S.I.H.	S.I.H.	C.H.	C.H.	S.I.H.	M.I.H.	M.I.H.	I.H.
	.2	M.I.H.	M.I.H.	S.I.H.	S.I.H.	M.I.H.	I.H.	I.H.
	.3	I.H.	M.I.H.	M.I.H.	M.I.H.	I.H.
Immune serum, .3 cc.	.04	S.I.H.	M.I.H.	S.I.H.	C.H.	M.I.H.	M.I.H.	C.H.	I.H.
	.06	S.I.H.	I.H.	S.I.H.	S.I.H.	I.H.	I.H.	C.H.	I.H.
	.08	M.I.H.	M.I.H.	S.I.H.	S.I.H.
	.1	I.H.	I.H.	M.I.H.	S.I.H.
	.15	I.H.	M.I.H.
	.18	M.I.H.
	.2	I.H.
	.3

*C.H. = Complete hemolysis; S.I.H. = Slight inhibition of hemolysis; M.I.H. = Marked inhibition of hemolysis; I.H. = Inhibition of hemolysis.

The antigen proved fairly satisfactory and .08 cubic centimeter was determined as the antigenic unit, although with Sera 4 and 7 the degree of complement fixation was slight but definite.

TABLE 3.

Fixing power of diphtheria immune serum.

Received from Dr. A. P. Hitchens, of Glenolden; horse No. 1428. Bleeding by G. on Dec. 11, 1911; serum received and tests made on Dec. 15, 1911. This serum contained 250 units of antitoxin per cubic centimeter.

Immune Serum, cc.	Amount of Ant., cc.	Ant. No. 1.	Ant. No. 2.	Ant. No. 3.	Ant. No. 4.	Ant. No. 5.	Ant. No. 6.	Ant. No. 7.	Ant. No. 8.
.02	.08	S.I.H.	S.I.H.	C.H.	C.H.	S.I.H.	C.H.	C.H.	S.I.H.
.04	.08	M.I.H.	M.I.H.	C.H.	C.H.	M.I.H.	C.H.	S.I.H.	M.I.H.
.06	.08	I.H.	M.I.H.	S.I.H.	C.H.	I.H.	C.H.	S.I.H.	M.I.H.
.08	.08	I.H.	I.H.	S.I.H.	S.I.H.	I.H.	S.I.H.	M.I.H.	I.H.
.10	.08	I.H.	I.H.	M.I.H.	S.I.H.	M.I.H.	M.I.H.	I.H.
.15	.08	M.I.H.	M.I.H.	M.I.H.	I.H.
.18	.08	I.H.	I.H.	I.H.	I.H.
.2	.08	I.H.	I.H.	I.H.

TABLE 4.

Fixing power of diphtheria immune serum.

Received from Dr. Ranzohoff, of New York Board of Health; horse No. 448; bleeding Dec. 15, 1911; serum received and tests made Dec. 18, 1911. This serum contained 600 units of antitoxin per cubic centimeter.

Immune Serum, cc.	Amount of Ant., cc.	Ant. No. 1.	Ant. No. 2.	Ant. No. 3.	Ant. No. 4.	Ant. No. 5.	Ant. No. 6.	Ant. No. 7.	Ant. No. 8.
.02	.08	C.H.	C.H.	C.H.	C.H.	S.I.H.	C.H.	C.H.	C.H.
.04	.08	S.I.H.	C.H.	C.H.	C.H.	S.I.H.	C.H.	S.I.H.	C.H.
.06	.08	S.I.H.	S.I.H.	S.I.H.	C.H.	M.I.H.	S.I.H.	S.I.H.	S.I.H.
.08	.08	M.I.H.	S.I.H.	M.I.H.	S.I.H.	M.I.H.	S.I.H.	M.I.H.	M.I.H.
.1	.08	I.H.	M.I.H.	M.I.H.	S.I.H.	I.H.	M.I.H.	M.I.H.	I.H.
.15	.08	I.H.	I.H.	I.H.	M.I.H.	I.H.	I.H.	I.H.	I.H.
.18	.08	I.H.	I.H.	I.H.	I.H.	I.H.
.2	I.H.

TABLE 5.

Fixing power of diphtheria immune serum.

Received from Dr. Alexander Company, of Marietta, Pa.; horse No. 197; bleeding Dec. 16, 1911; serum received and tests made Dec. 18, 1911. This serum contained 1,000 units of antitoxin per cubic centimeter.

Immune Serum, cc.	Amount of Ant., cc.	Ant. No. 1.	Ant. No. 2.	Ant. No. 3.	Ant. No. 4.	Ant. No. 5.	Ant. No. 6.	Ant. No. 7.	Ant. No. 8.
.02	.08	S.I.H.	S.I.H.	C.H.	S.I.H.	S.I.H.	S.I.H.	I.H.	I.H.
.04	.08	M.I.H.	M.I.H.	S.I.H.	S.I.H.	M.I.H.	S.I.H.	I.H.	I.H.
.06	.08	I.H.	M.I.H.	S.I.H.	M.I.H.	I.H.	M.I.H.
.08	.08	I.H.	I.H.	M.I.H.	I.H.	I.H.	I.H.
.1	.08	I.H.	I.H.	I.H.	I.H.
.15	.08	I.H.
.18	.08
.2	.08

It is noted at once that complement-fixing bodies are present in diphtheria immune sera. That these bear a close relation to the antigens of diphtheria bacilli is to be noted in the above and following tables, where the three sera were tested with different antigens.

TABLE 6.

Control tests.

Antigen of alcoholic extract of luetic liver which was antigenic with syphilitic antibody in .2 cubic centimeter of a 1:3 dilution; anticomplementary .8 cubic centimeter.

Amount of Ant., cc.	Amount of Serum, cc.	Mulford Serum.	New York Serum.	Alexander Serum.
.2	.04	C.H.	C.H.	C.H.
.2	.06	C.H.	C.H.	C.H.
.2	.08	C.H.	C.H.	C.H.
.2	.1	C.H.	C.H.	C.H.
.2	.15	S.I.H.	C.H.	C.H.
.2	.18	S.I.H.	C.H.	C.H.
.2	.2	M.I.H.	C.H.	S.I.H.
.2	M.I.H.	S.I.H.	S.I.H.

TABLE 7.
Control tests.

Antigen of acetone-insoluble lipoids which was antigenic in amounts of .1 cubic centimeter with syphilitic serum; anticomplementary in amounts of .45 cubic centimeter.

Amount of Ant., cc.	Amount of Serum, cc.	Mulford Serum.	New York Serum.	Alexander Serum.
.1	.04	C.H.	C.H.	C.H.
.1	.06	C.H.	C.H.	C.H.
.1	.08	C.H.	C.H.	C.H.
.1	.1	C.H.	C.H.	S.I.H.
.1	.15	S.I.H.	C.H.	M.I.H.
.1	.18	M.I.H.	S.I.H.	I.H.
.1	.2	M.I.H.	M.I.H.	I.H.

The following will be noted :

1. There is present in diphtheria immune sera, in addition to the antitoxin, a substance capable of binding complement with the specific antigens.

2. There is no relation between the quantity of antitoxin present and the complement-fixing power of the serum.

3. Of the eight cultures used as antigens three were of virulent diphtheria bacilli. Culture No. 3, Park's Bac. No. 8, is used extensively in the manufacture of antitoxin. The quantity of antitoxin produced is dependent to an extent upon the virulence of the organism; the occurrence and degree of complement fixation does not bear any direct relation to the type or virulence of the culture.

2. Opsonins in diphtheria immune serums :

The quantity of opsonin in the serum of normal individuals for diphtheria bacilli is quite low. With normal serum I have found about four to ten per cent of the leucocytes become phagocytic. Normal horse serum is more readily

phagocytic, about twenty-six per cent of the leucocytes being found active.

The opsonic indices of the three immune serums were determined with fresh emulsions of the eight different cultures with the following results:

TABLE 8.
Opsonic indices; diphtheria immune serums.

Immune Serum.	Antitoxin Content Units per cc.	Cult. No. 1.	Cult. No. 2.	Cult. No. 3.	Cult. No. 4.	Cult. No. 5.	Cult. No. 6.	Cult. No. 7.	Cult. No. 8.
Mulford . .	250	1.2	1.6	1.3	1.4	1.1	1.0	1.2	1.4
New York .	600	2.1	1.0	1.5	2.0	1.9	1.2	1.8	1.6
Alexander .	1,000	3.2	2.1	1.9	2.0	2.6	2.0	2.5	2.4

These determinations were made in from one to two days after receiving the serums. No preservatives were added and serums were kept on ice.

1. It will be noted that the quantity of opsonin is increased in the immune serums and bears a direct relation to the antitoxin content.

2. No specific relations are noted between the opsonin present and the different cultures of diphtheria bacilli.

3. There is no relation between the opsonin content and degree of complement fixation.

Conclusions.

The prophylactic and curative properties of diphtheria immune serum depends largely upon the presence of receptors of the first order. Such serum is not bacteriolytic and the administration of the serum is primarily to neutralize the toxins. These single receptors have probably no direct influence upon the bacilli themselves. However the local lesions improve and the bacilli disappear following the administration of the serum and this action may be due in part to the opsonins produced by the patient's own cells, aided by that introduced in the serum. However the administration of serum has no influence upon the bacilli of "carrier"

cases and I have found that bacterial treatment with auto-genous vaccines, in the hope of raising the opsonic index, has been generally unsuccessful in twenty-four cases. The complement-binding substance found in these serums had a direct affinity for the specific antigens. The quantity of this substance bore no relation either to virulence of the antigen or to the quantity of first degree receptors present. It is practically certain that they are not bacteriolytic receptors.

STREPTOCOCCUS IMMUNE SERUM.

The exact nature of streptococcus immune serum is not known. The serum is neither bacteriolytic nor antitoxic or but feebly so, and its value in the treatment of streptococcus infections depends probably upon opsonins. Bordet noted that phagocytosis of streptococci was feeble and sluggish and believed such to be due to a negative chemiotactic influence and that this influence is removed upon the administration of the serum. We have studied the question of streptococcus antibodies in immune serums and scarlet fever¹ and will give here but a resumé of the work as relates to this particular study.

1. Complement fixation.

Organisms and Immune Serums.

In all of the following experiments the same cultures and immune serums were used. Accordingly reference will be made to culture and serums by number only. For instance, Antigen 1 means that it was made of Streptococcus Culture 1, and Immune Serum 1 means that it was produced by injections of Streptococcus Culture 1, and so on through opsonins and agglutinins.

A. No. 1: Streptococci secured from scarlet fever patients. A horse was immunized (Immune Serum 1) by three injections of streptococci killed by exposing to 60° C. for one hour; the next two injections were of attenuated organisms after exposure to 50° C. for thirty minutes, and

the last injection preceding bleeding was of living cultures. All injections were subcutaneous.

No. 2: A streptococcus secured by blood culture from a case of scarlet fever. A rabbit was immunized (Immune Serum 2) by six intravenous injections, at intervals of a week, of organisms exposed to 60° C. for one hour.

No. 3: A streptococcus from a case of septicemia, obtained from Dr. A. P. Hitchens. A rabbit was immunized (Immune Serum 3) by six intravenous injections of organisms exposed to 60° C. for one hour.

No. 4: A streptococcus, isolated by Fillin from a case of septicemia, and obtained from the American Museum of Natural History, New York. A rabbit was immunized (Immune Serum 4) by six intravenous injections of organisms exposed to 60° C. for one hour.

No. 5: A streptococcus isolated from a non-scarlatinal tonsillitis. A rabbit (Immune Serum 5) was immunized by six intravenous injections of organisms exposed to 60° C. for one hour.

The injections were given every seven days and the serum tested from time to time by bleeding from the ear. At the end of seven weeks the rabbits were killed and the serum stored in sterile tubes in the refrigerator without the addition of preservatives. Normal horse serum was secured from two healthy animals and normal rabbit serum from six large healthy rabbits.

B. The antigens were prepared in the same manner as those of diphtheria bacilli. These were found antigenic in amounts of .1 cubic centimeter and became anticomplementary in amounts of over .18 cubic centimeter.

Six streptococcus immune serums were then tested separately with the five antigens. Five of these were prepared as already given; the sixth was a polyvalent serum received through the kindness of Dr. A. P. Hitchens. A fairly well-marked degree of complement fixation resulted, and these showed on the whole that in the higher dilutions of the immune serums a specific relation exists between immune body and its specific antigen. In lower dilutions of serum

no differentiation resulted with the various antigens. As far as this present study is concerned the most important finding was that of complement-fixing bodies.

The following two tables are included to show the lack of relation between complement fixation and opsonin and agglutinin content of streptococcus immune serum (compare with Tables 11 and 12):

TABLE 9.
Fixing power of Serum 1.

Amount of Serum, cc.	Antigen, cc.	Strepto-coccus 1.	Strepto-coccus 2.	Strepto-coccus 3.	Strepto-coccus 4.	Strepto-coccus 5.
.05	.1	S.I.H.	S.I.H.	C.H.	C.H.	C.H.
.08	.1	M.I.H.	S.I.H.	C.H.	C.H.	C.H.
.1	.1	I.H.	M.I.H.	C.H.	C.H.	S.I.H.
.15	.1	I.H.	I.H.	S.I.H.	S.I.H.	S.I.H.
.2	.1	I.H.	I.H.	S.I.H.	M.I.H.	M.I.H.

It will be noted (Table 9) that complement fixation is most marked with the Scarlet fever streptococcus antigens 1 and 2.

TABLE 10.
Fixing power of polyvalent serum.

Amount of Serum, cc.	Antigen, cc.	Strepto-coccus 1.	Strepto-coccus 2.	Strepto-coccus 3.	Strepto-coccus 4.	Strepto-coccus 5.
.05	.1	C.H.	C.H.	S.I.H.	C.H.	M.I.H.
.08	.1	C.H.	C.H.	I.H.	C.H.	I.H.
.1	.1	S.I.H.	S.I.H.	I.H.	C.H.	I.H.
.15	.1	S.I.H.	S.I.H.	I.H.	S.I.H.	I.H.
.2	.1	S.I.H.	S.I.H.	I.H.	M.I.H.	I.H.

It will be noted in Table 10 that fixation is best marked with Streptococcus 5, isolated from a case of streptococcic

tonsillitis; then with *Streptococcus* 3, a culture sent me by Dr. Hitchens. The serum also contained antibodies for scarlet fever streptococci as demonstrated by the reactions with 1 and 2. This would tend to show that each streptococcus produced its own antibody.

2. Opsonins:

The streptococco-opsonic indices of the six immune serums, including the polyvalent serum, were determined with the five streptococcus cultures. The following table gives the results of these determinations. The polyvalent serum was the result of more than six injections:

TABLE II.
Opsonic indices after six injections of streptococci.

Immune Serum.	Strepto- coccus 1.	Strepto- coccus 2.	Strepto- coccus 3.	Strepto- coccus 4.	Strepto- coccus 5.
Horse Serum 1	3.2	3.2	1.1	1.0	1.1
Rabbit 2.	1.2	1.2	0.6	0.8	0.9
Rabbit 3.	0.6	0.6	1.6	0.8	0.9
Rabbit 4.	0.2	0.2	0.37	1.4	0.32
Rabbit 5.	0.8	0.8	0.85	0.7	1.7
Polyvalent serum	2.8	2.8	3.3	4.1	3.6

These results indicate a more or less specific relation between opsonins and the cultures used in the process of immunization. There is, however, apparently no relation between the degree of complement fixation and opsonin content.

3. Agglutinins:

The agglutinin content was determined in each of the six immune sera with the five different cultures. Agglutinins are present in streptococcus immune serums, but the amount varies within wide limits and they are not specific for the

strain of streptococcus used in the process of immunization and are of no value in differentiating streptococci. The study also showed the absence of any direct relation between opsonin and agglutinin content as the following table will show :

TABLE 12.
Showing results of agglutination of streptococci by immune serums.

Immune Serum I.

Streptococcus.	Dilutions.							
	1/10	1/15	1/20	1/25	1/30	1/40	1/100	1/140
1.....	+	+	+	+	+	+	+	-
2.....	+	+	+	+	+	+	+	-
3.....	+	+	+	+	+	-	-	-
4.....	+	+	+	-	-	-	-	-
5.....	+	+	+	+	+	-	-	-

Polyvalent Serum.

1.....	+	+	+	+	-	-	-	
2.....	+	+	+	+	-	-	-	
3.....	+	+	+	+	+	+	-	
4.....	+	+	-	-	-	-	-	
5.....	+	+	+	+	-	-	-	

Comparison with Table 10 shows the absence of any relation between agglutinins and complement-fixation substances.

Conclusions.

In streptococcus immune serum are a number of different antibodies, mainly agglutinins, opsonins, and a complement-fixing substance. Such serums are apparently free of anti-toxin or receptors of the first order and it is doubtful if they are bactericidal although the latter point is difficult to decide,

for such tests require careful relations between amboceptor and complement. I regret not having performed precipitin tests with these serums and it is probable that a relation exists between the occurrence and degree of complement fixation and precipitin content. Just what is the nature of this complement-binding substance is difficult to state. The precipitins belong strictly to the second order of receptors and cannot be activated by the addition of normal serum after having been inactivated. There are no hemolytic immune bodies present in streptococcus serums and evidence is lacking as to bactericidal amboceptors. Therefore the complement-binding properties are difficult to explain. There is apparently no relation between the complement-binding bodies, opsonins, or agglutinins in streptococcus immune serums.

CHOLERA AND TYPHOID IMMUNE SERUMS.

These immune serums were examined quantitatively for complement-fixing substances, agglutinins, opsonins, and bacteriolysons. Since both serums are of the same nature they are considered together.

1. Complement-fixation tests:

Immune sera: These were prepared by immunizing a series of rabbits by intravenous injections of salt solution emulsions heated to 58° C. for one hour.

Antigens: These were prepared in the same manner as the diphtheria and streptococcus antigens. The antigenic and anticomplementary values of each antigen were as follows:

Cholera: Antigenic .02 cubic centimeter; anticomplementary .25 cubic centimeter.

Typhoid: Antigenic .06 cubic centimeter; anticomplementary .15 cubic centimeter.

With an antigen of alcoholic extract of luetic liver which was antigenic for syphilitic antibodies in amounts of .2 cubic centimeter of 1:3 dilution, the cholera and typhoid immune serums yielded absolutely negative results up to .25 cubic

centimeter of immune serum, using the exact unit of complement.

To still further test the specific relation between antibody and antigen, both cholera and typhoid immune serums were tested with the eight diphtheria, a staphylococcus and two of the streptococcus antigens, with uniformly negative results.

Conversely the Hitchens, New York, and Alexander diphtheria immune serums were tested with the cholera and typhoid antigens with negative results.

2. Agglutinins:

With the ordinary macroscopic technic the following results were secured:

Cholera serum: Agglutinates homologous culture in dilution of 1:950.

Typhoid serum: Agglutinates homologous culture in dilution of 1:1,500.

3. Opsonins:

These determinations were made with the fresh serums and repeated twice with the following results:

Cholera opsonic index: 2.12

Typhoid opsonic index: 3.81

4. Bactericidal tests:

Neisser's test-tube method was employed. The technic requires sterile vessels, diluting fluids and serums, and general caution. Fixation of complement in a test-tube containing the bacterial emulsions, complement, and amboceptor may be caused by an excess of amboceptor because in such a mixture the complement is not only bound by the attached amboceptor but also by free amboceptor, and a portion of the bound amboceptor may therefore lack complement to exert its bactericidal effects. Therefore for reliable bactericidal tests the proportions of amboceptor and complement must be exact, for if one or the other of these is in excess no bactericidal action occurs. The method will probably not become popular for diagnostic purposes.

The mixtures of bacteria, complement, amboceptor, and diluting fluid were incubated for three hours and then plated with neutral plain agar-agar. Plates were incubated for twenty-four hours and then counted. The following results were noted:

Cholera: Dilutions of the immune serum were made from 1:100 to 1:810,000. The 1:100 dilution showed one thousand five hundred colonies; 1:150 showed eight thousand five hundred colonies; dilutions over 1:9,000 showed more than fifty thousand colonies per plate.

Typhoid: The same dilutions as with the cholera serum were made. The 1:100 dilution showed one hundred and twenty colonies per plate; the 1:500 dilution showed nine hundred and fifty colonies; the 1:3,200 showed thirty-eight thousand colonies, and dilutions over 1:6,400 showed over fifty thousand colonies per plate.

Proper controls of cultures, sterility of serums, inactive serums and without complement were made.

Conclusions.

1. There was no relation between the degree of complement fixation and opsonins, agglutinins or bacteriolysins in either the cholera or typhoid immune serums. Thus the cholera serum gave the best complement-fixing result but contained less of the other antibodies.

2. Judging from the results of these two serums there is some relation between the opsonin content and bacteriolysins.

DISCUSSION.

Practically all of the now known antibodies, lysins, agglutinins, opsonins, and antitoxins may be found in normal serum in varying amounts and each have more or less distinctive characteristics. It is doubtful if precipitins exist in normal serum. The presence of these antibodies in normal serum simply proves that somewhere in the animal are receptors which have an affinity for the antigen in question

and that normally there is a varied but moderate overproduction of these receptors which are thrust off into the blood. As a result of immunization there results an enormous overproduction of some particular receptor which overshadows all others present. Thus in diphtheria immune serum the antitoxin is far in excess of the opsonins.

And yet it appears that a single antigen may stimulate the production of various antibodies. Thus in the diphtheria immune serums the opsonin content is far above the normal. Likewise there is produced a complement-binding substance. Streptococci stimulate the production of opsonins and agglutinins and cholera and typhoid antigens produce agglutinins, bacteriolysins, opsonins and complement-fixing bodies far in excess of those found in normal serums. Therefore it seems apparent that the system of receptors in the animal economy is not at all a staple one, but easily disturbed through the influence of a simple antigen.

As a general rule the various antibodies are considered more or less distinct and separate from each other, but there are exceptions to this rule. The complement-fixing bodies showed no relation at all to the other antibodies studied in our serums. Each antibody bears a direct relation with its antigen and in this manner facilitates diagnosis. Receptors of the first order, antitoxins, produced as a result of stimulation by a soluble toxin are probably the best marked of all. There is, however, a more or less direct relation between the antitoxin and opsonin content of the diphtheria immune serums and likewise between the opsonins and bacteriolysins in the cholera and typhoid serums. Essentially these antibodies are different and easily separated, but in serums which are prophylactic and curative these antibodies, antitoxins, opsonins, and bacteriolysins bear a relation to each other. Levaditi identifies the immune opsonins and lytic amboceptors. On the other hand, the agglutinins and precipitins are probably not antibodies in the strict sense of being directly inimical to the antigen, and their presence therefore does not mean increased resistance in virtue of themselves, although their presence in the serum does indicate

heightened resistance through the agency of other antibodies. Thus the agglutinins to be found in the serum of a person immunized with typhoid bacteria may be considered an indication of his resistance, although the agglutinin itself has not the power to lower the virulence of or destroy the typhoid bacillus. In the cholera and typhoid immune serums there is an apparent relation between the agglutinins and precipitins and it is probable that they are quite similar immune bodies. Between agglutinins, precipitins, and lysins there is no relation whatsoever. Thus a serum rich in agglutinin may have poor bactericidal powers or vice versa.

The relation of complement fixation to the various receptors has been especially interesting. Thus with receptors of the third order it is claimed that a bactericidal amboceptor produced by immunization came to be recognized by complement-fixation tests, whereby bacteria supplied with the amboceptor are able to bind hemolytic complement and thereby show that the bacteria have combined with a bactericidal amboceptor. Neisser has not been able to substantiate these claims as made originally by Borget and Gengou and in the present study it will be noted that the cholera serum gave better complement fixation than the typhoid serum but was weaker in its bactericidal action. I do not think that the ordinary complement-fixation technic will suffice to measure the bactericidal power of an immune serum, and in this connection one must bear in mind the great difficulty of studying such a serum either *in vivo* or *in vitro* by any methods on account of the close counteracting influences of complement and amboceptor. Attention has already been drawn to the fact that complement may not only be fixed by amboceptor attached to the bacterial cell but also by free amboceptor and the complement thus bound to free amboceptor means so much less available complement to exert a bactericidal effect. For the same reasons the injection of bacteriolytic serum in the cure of disease is of problematical value because the good effects depend so largely upon the amount of complement which may be present.

All of the immune serums which I have examined contained some body capable of fixing hemolytic complement. This is the basis for such tests aiming to detect either antigen or antibody by complement fixation. But the nature of the complement-binding substances are difficult to understand. Thus diphtheria immune serum certainly contains no lysins or receptors of the third order and yet such serums bind complement with specific antigens quite readily. It will prove most interesting to work out the various antibodies in syphilitic immune serum and determine their relation to complement fixation now that Noguchi has devised a method for growing *Treponema pallidum* in pure culture. Not infrequently these tests apparently show the largest amount of antibody present at a time when the patient clinically is gradually developing severer symptoms and signs of syphilis, whereas strictly speaking the largest amount of antibody should be found at the height and decline of the infection. With these considerations combined with the lack of relation between complement fixation and bacteriolysins it would appear that a special body is stimulated and thrown into the blood stream in the nature of a reacting substance, a "reagin," capable of binding hemolytic complement and not a true antibody in the sense of being prophylactic against or destructive to the antigen.

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PRIMARY CANCER OF THE LUNG.*

A. T. HENRICI.

(*Magee Pathological Laboratory, Mercy Hospital, Pittsburgh, Pa.*)

Primary carcinoma of the lungs is a rare affection. Its incidence may be roughly indicated by the statistics of Reinhardt, Fuchs and Passler. Reinhardt,¹ in eight thousand seven hundred and sixteen autopsies at the Dresden City Hospital, found five hundred and forty-five cases of carcinoma, of which five were primary in the lungs. Fuchs² found in the Munich records eight cases of primary lung cancer in twelve thousand three hundred and seven autopsies. Passler³ records, out of one thousand cases of malignant disease from the Breslau Pathological Institute, four primary sarcomas and sixteen primary carcinomas of the lung. It must be borne in mind that it is difficult to differentiate between primary carcinomas arising in the lung and those of bronchial origin, and that probably many tumors of the latter type are included in the statistics quoted above. Rolleston and Trevor⁴ conclude that these figures for lung cancers are too high, and state that there is no reason for believing that carcinoma is more frequent than sarcoma. Out of three thousand nine hundred and eighty-three autopsies at St. George's Hospital they found three sarcomas and no carcinomas primary in the lungs.

While there have been a large number of cases reported, not all have been undoubted cases of primary cancer of the lung, and in many cases the reports were incomplete. From a critical examination of the literature up to 1896 Passler³ gathered the data of seventy cases in which the reports were complete enough to be of value, and to these added four from the Breslau Pathological Institute. From a partial review of the literature since that time I have been able to collect the reports of ten additional cases which, with the one here reported, makes a total of eighty-five from which statistical data concerning the type of tumor can be drawn. The

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ten cases were reported by Ernst,⁵ Ribbert,⁶ Delorme,⁷ Brecken,⁸ Ennet,⁹ Herrmann,¹⁰ Parrow,¹¹ and Kaminski¹² (one case each), and Lammerhirt¹³ (two cases). The last five were from the Greifswald Pathological Institute.

Of these eighty-five cases many are probably and some certainly of bronchial origin, but no attempt has been made to separate them. In the following table these cases have been classified according to the histological type of tumor present.

Squamous celled.....	12
Cylindrical celled.....	24
Cubical celled.....	1
Transitional cells	1
Polygonal cells.....	9
Polymorphous cells	11
"Large epithelial cells".....	4
Scirrhus.....	3
Cancer, but the type not stated	12
No microscopic report.....	8

Among the squamous celled tumors is included one in which it was stated that the cells were "similar to alveolar cells." Four of these tumors showed keratinization. Of the cylindrical celled cancers three are described as forming gland structures. Several of the polygonal and polymorphous celled growths are stated to have shown in places cylindrical or squamous cells, but a more indefinite type of cell predominated.

As to the etiology of lung cancers but little more can be said than for the etiology of malignant growths in general. The case reported by Ribbert⁶ was associated with fibrosis of the lung, together with other signs of a previous inflammatory process, and he calls attention here to his well-known theory of the origin of cancer, which is apparently supported by this instance. The cases of Grant,¹⁴ Lawrence and Suckling,¹⁵ Friedlander,¹⁶ and Ennet⁹ were associated with pulmonary tuberculosis. It has been stated by Wieber¹⁸ and restated by Osler that cancer of the lung is remarkably frequent among the cobalt miners of Schneeberg. It is possible that here a type of pneumonokoniosis is a factor in

the etiology. The case reported in this paper was associated with marked anthracosis. But both tuberculosis and anthracosis are so common, and cancers of the lung so rare, that an etiological relationship could hardly be considered.

It might be remarked, concerning the reported cases of cancer occurring at Schneeberg, that definite histological reports of these tumors are not forthcoming and that some investigators suggest that mediastinal tumors or even cases of Hodgkin's disease have been included in the series.¹⁹

There are three possible points of origin for the tumors of the lung; the alveolar epithelium of the air sacs, the bronchial epithelium and the epithelial lining of the bronchial mucous glands. Concerning the latter but little can be said and it may possibly be the point of origin of some of the apparent adeno-carcinomata. About the other two, however, considerable polemical literature has accumulated. Some of the tumors mentioned above have undoubtedly arisen from the bronchial mucosa remaining isolated in the bronchi. Others, by their anatomical situation, and the fact that they have infiltrated the lung along the bronchial tree, have left but little doubt as to their bronchial origin. But the majority, on account of their large size or wide dissemination, offer nothing in the gross appearance to indicate from what structures they have arisen.

As to the cylindrical celled tumors, it is generally conceded that they arise from the epithelium of the bronchial mucosa, or possibly from the bronchial mucous glands. The fact is to be considered, however, that the embryonic type of alveolar epithelium is cylindrical in character. And the possibility also presents itself that in atelectatic lung tissue the epithelium may undergo a true metaplasia to cylindrical epithelium and, having been rendered functionless, acquires the "habit of growth," and produce a tumor.

The origin of the squamous celled tumors is still more difficult to explain. Some of them have been of undoubted bronchial origin, but in others this is not so clear. Siegal, Perls, Grunwald and Wechselsmann believe that they arise from the alveolar epithelium. Tillmann speaks of a tumor

composed of cells "similar to normal alveolar epithelium," and to these tumors Grunwald has applied the term "alveolar epithelioma." But it is a far cry from simple alveolar epithelium to a structure composed of stratified squamous cells showing intercellular bridges, forming types of epithelial pearls, and in some cases producing keratin. On the other hand, to explain an origin from the bronchial mucosa there must be called into play a process of metaplasia. It is interesting in this connection to note that such a process has been noted in the bronchi, in the absence of any malignant change, by Friedlander, Curt Wolf, and recently by S. R. Haythorn. The latter observer found areas of metaplasia to squamous epithelium in the bronchial mucosæ of three lungs from this laboratory, all being cases of pneumonia. He also found, in sections from the lung described below, areas of metaplasia in the bronchi, the columnar cells being replaced by squamous cells similar to those forming the tumor. Haythorn does not consider that there is a direct metaplasia of the columnar cells to squamous cells, but that the latter arise from the deeper epithelial cells after the columnar cells have been destroyed or lost during an inflammatory process. On account of these observations it is probable that the metaplasia is the primary process, and that the tumors arise from such metaplastic epithelium. This is the explanation which Ernst⁵ accepts for his case.

A third possibility may be briefly mentioned, that some of those tumors are in reality endotheliomata. This is the view of Schottelius.

The intermediate types of cancer offer but little more in their microscopic structure than they do in the gross appearance towards solving the question of their origin. We must agree with Ribbert,⁶ that "it is impossible, in cases of far-advanced carcinoma, to determine anything of their origin."

The case here reported is one of non-keratinizing squamous celled carcinoma arising in and remaining isolated to the right lung. The patient, Mr. M. C., aged sixty-two, was admitted to the service of Dr. C. O. Goulding in the Mercy Hospital on Aug. 1, 1911, in an intoxicated

condition. The family history was not important. He had gone through the usual diseases of childhood, and had repeatedly been ill following intoxication. The patient had been drinking heavily for some days before admission. The day following his admission his temperature rose to 102°. On examination the right chest was found on percussion to be flat from the clavicle to the fourth rib anteriorly, with bronchial breathing over this area. He was coughing and expectorated blood-tinged sputum. For a week the temperature varied between 98° and 101°, being low in the morning and high in the evening. At the end of a week rales appeared at the right apex. The temperature gradually subsided, but varied from 97° to 100° for his remaining illness. The consolidated area never became resonant. The patient grew very weak and developed a marked pallor, but never appeared cachectic. For two weeks before death he was delirious, and had no control of his sphincters. Sputum examinations from time to time revealed the presence of pneumococci and streptococci, tubercle bacilli were not found, and cancer tissue, if present, could not be recognized. Death occurred on September 4. A clinical diagnosis of unresolved pneumonia of the right upper lobe was made.

At autopsy a small portion of the right lung was found to be adherent to the sternum. In the left pleural cavity there were many old adhesions, and a small quantity of blood-stained fluid. The upper and middle lobes of the right lung were firmly adherent to the parietal pleura and the pericardium. The left lung showed a marked marbling of the pleura due to advanced anthracosis. On section the upper lobe was seen to be rather moist and crepitated throughout. The lower lobe contained a moderate amount of grayish fluid. The bronchi contained a mucoid exudate. Over the upper and middle lobes of the right lung there was a dense, plate-like organized exudate. Over the upper third of the lower lobe there was a fine, sand-like fibrinous exudate. The anterior surface at the lower two-thirds was free from exudate. On the posterior surface there was a thin, very vascular membrane. The upper and middle lobes and the upper part of the lower lobe were completely solidified. The lower lobe crepitated. On section the upper lobe was seen to be replaced by an enormous mass composed of a pinkish, cream-colored tissue of a pasty consistency. Posteriorly this mass was bounded only by the thickened pleura and about two centimeters of blackened atelectatic lung.

In two places the mass extended forwards to the pleura. The cut surface of the tumor measured 9.5 by 6 centimeters. In the middle lobe there were two smaller areas of similar, soft friable tissue. The lung tissue of the lower lobe was of a grayish red color; the lower border was edematous. The only remaining normal tissue in the areas of new growth were some bands of connective tissue and some blood vessels. In the main bronchus of the upper lobe there were soft papillary projections into the lumen. The peribronchial lymph nodes of both lungs showed marked anthracosis, but no necrosis or evidence of new growth. One gland at the bifurcation of the trachæ was calcified.

The other autopsy findings were of minor interest. The aortic and mitral valves were sclerosed; there was sclerosis of the aorta and coronaries. There were multiple hemorrhages into the spleen and the submucosa of both the large and small bowels. The left lateral sinus contained a firm, white thrombus, which was adherent. There was a chronic inflammation of both middle ears. The testicles were fibrosed. There were no new growths found in any part of the body, save the right lung.

The microscopical examination of the tumor showed it to be composed of cell masses having a general arrangement suggestive of an alveolar structure. Many of these masses were quite solid, but the majority of them contained an open space in the center filled with necrotic material. Here and there large, irregular arborescent or serpentine masses of tumor cells were formed, surrounded by a necrotic material. The stroma was thin and the relationship of the tumor cells to it was quite definite. Lying upon the basement membrane of the stroma there was a single layer of columnar cells, but the layer above this consisted of smaller, round and irregular cells, and above this layer the cells were squamous in type. As a rule the tumor cells formed only a thin layer, ten or fifteen cells deep. The upper ones were definitely squamous in type, showing intercellular bridges, and forming here and there small concentric whorls, which seldom consisted of more than five or six elements. No keratinization could be demonstrated, although a large number of sections

were examined, using not only the usual stains but also Gram's, which, as Ernst⁵ has pointed out, is a selective stain for keratin. The cells had large, deeply staining nuclei, and a rather indistinct, faintly staining protoplasm. Mitotic figures were numerous.

As has been mentioned, the stroma was very delicate, consisting of a few fibrils supporting the cell masses. Sections stained with Van Gieson's and Mallory's stains showed the greater part of the stroma to be composed of white fibrous tissue, but sections stained by Weigert's elastic tissue showed the presence of fibers of the latter type also. This fact, together with the alveolar structure of the tumor masses which became more apparent in these sections, suggested that the original stroma of the lung had been retained, and that the tumor had grown into the lung along the alveolar walls. But the alveolar structures were usually larger than lung alveoli, and they were so indefinite and inconstant as to preclude the possibility of drawing any definite conclusions of their origin. The open spaces in the center of the masses, however, were clearly not due to necrosis of the tumor, as the cells bordering them did not show signs of destruction.

The stroma carried only a few small capillaries and, when compared with the amount of tissue supplied, appeared quite inadequate. The necrotic portions of the tumor showed the presence of a number of polymorphonuclear leucocytes and here and there an infiltration of lymphocytes into the stroma. About the tumor there was seen a border of compressed and fibrosed lung tissue, showing a considerable infiltration of lymphoid cells. A moderate amount of anthracosis was present.

Sections of the nodules in the bronchi showed an invasion of the bronchial wall by tumor from the surrounding tissue. Sections of the peribronchial lymph glands failed to show any evidence of metastases.

There are several points about the case that merit discussion. Definite conclusions as to the origin of the tumor cannot be drawn from gross appearances on account of the

wide involvement of the growth obliterating all structures. In the microscopic examination of the specimen there is only one point which is helpful; namely, the finding of areas of metaplasia of columnar to squamous epithelium in the bronchi, unassociated, however, with any malignant change. It is quite probable that the tumor arose from epithelium of this nature.

The absence of keratinization is of interest. Although Ernst⁵ lays especial stress on the formation of keratin in this type of lung tumor as essential to the diagnosis, it is to be noted that this process occurred in only four of the twelve squamous celled cancers tabulated above. Moreover, non-keratinizing squamous celled cancers are frequently noted in other organs as, for instance, in the cervix uteri and the bladder. In the case here reported the presence of definitely squamous cells with whorl formation and the presence of intercellular bridges leaves but little doubt as to the diagnosis.

The apparent alveolar structure, as though the tumor had invaded the lung parenchyma along the alveolar walls, seems to occur frequently in carcinomata of this organ. In eight of the eighty-five cases mentioned above such an arrangement was mentioned as being present. Passler observed in his third case that "the cancer fills the original alveoli and the alveolar septa form the stroma." The finding of elastic tissue fibers in the stroma of our tumor is additional evidence that such a process has also occurred here. Siegert,⁷ too, considers that cancers invade the lung in this manner and Ribbert⁶ agrees with him, although the latter author points out that there is another mode of invasion; namely, along the lymphatics of the alveolar septa.

Finally, it is noteworthy that so large and so cellular a tumor could be produced without forming distant metastases, even in the regional lymph nodes.

CONCLUSIONS.

1. Primary carcinoma of the lung is a rare affection, occurring approximately once in sixteen hundred autopsies.

2. Probably the majority of so-called cancers of the lung are in reality of bronchial origin.

3. Squamous celled cancers of the lung probably arise in the majority of cases from bronchial epithelium which has undergone a metaplasia.

4. Some of these tumors apparently invade the lung along the alveolar wall, retaining the alveolar septa as stroma.

[I here wish to express thanks to Dr. Oskar Klotz for his criticisms and aid in preparing this paper, and to Dr. S. R. Haythorn for the photomicrographs.]

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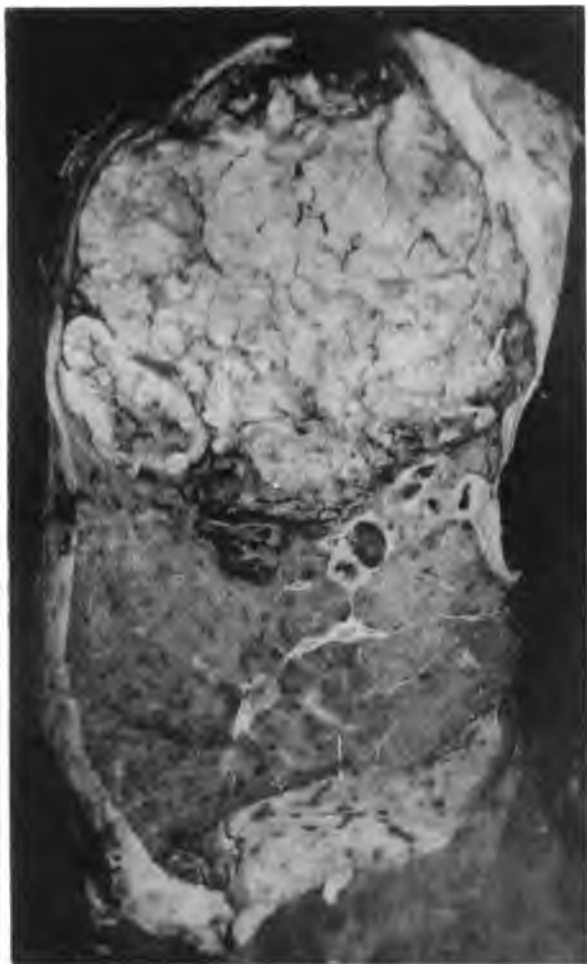
DESCRIPTION OF PLATE XVII.

FIG. 1. — Primary cancer of right lung.

FIG. 2. — Section at margin of cancer of lung, showing alveolar character with central lumen.

FIG. 3. — Cancer of lung showing squamous character of tumor cells, and apparent "perivascular" arrangement.

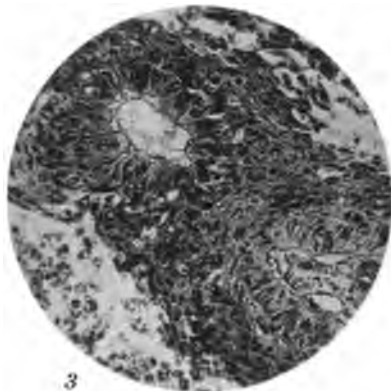
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ATROPHY OF THE PANCREAS AFTER OCCLUSION OF THE
PANCREATIC DUCT.*

LINDSAY S. MILNE, M.D.

AND

H. LEBARON PETERS, M.D.

(*Russell Sage Institute of Pathology, New York.*)

Ever since it has been known that pancreatectomy is followed by diabetes, opinions have been very varied as to the part the pancreas takes in metabolism. Nor is it yet settled whether its action is by virtue of an internal secretion, or by extracting or altering substances in the blood serum. Also, in spite of prolonged discussion it has not yet been decided whether the "islands" in the pancreas, first described by Langerhans in 1869, are the essential structures which control carbohydrate metabolism in the tissues.

One of the strongest morphological supports in favor of the "island theory," or independent existence of the "islands" and their functional importance, is based on the effect of ligature of the pancreatic duct. This operation has been described as producing a general atrophy of the acini of the pancreas, while the "islands" persist unaltered and no diabetes results. The subsequent removal, however, of the fibrous mass, representing the atrophied pancreas containing only "islands," is followed by diabetes.

The importance of establishing this has warranted the numerous works which have appeared confirming or contradicting it. All agree that ligature of the pancreatic duct, if reestablishment of the excretory channels is prevented, causes atrophy and eventually disappearance and fibrous tissue replacement of the pancreas acini.

In varying amount, however, small collections of epithelial cells more or less closely resembling "islands" do persist. These surviving clumps of cells seem to be capable of preventing diabetes, although Sauerbeck in his series of

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rabbits found that glycosuria did develop thirty days after the operation. What these surviving cells are, however, original Langerhans islands or pancreas acini, is still a subject of controversy.

The effects of occlusion of the pancreatic duct were observed in the following series of animals with rather varying results in each species; two guinea-pigs, six rabbits, five dogs, and twelve cats. In all these animals the tail portion of the pancreas was separated from the part attached to the duodenum and turned backwards so as to prevent reunion. The vascular supply of the pancreas was interfered with as little as possible and every care was taken to avoid infection of the pancreatic tissue.

The tendency to reëstablish the excretory channels of the pancreas is very marked and when this occurs sufficiently early large portions of the pancreas may be spared. In this way the statements of Hanseemann,¹ Lombroso,² and others that ligature of the pancreatic duct did not cause general atrophy of the pancreas acini may be accounted for.

In cats very uniform results were obtained. Atrophy of the acini begins very soon after the operation and in forty-eight hours is quite marked.

In ten days the cells lining the acini have become very much reduced in size and are commonly desquamated into the lumen of the tubule (Fig. 1). "Islands" can be readily observed and are apparently in the same condition as the acini, the shape and arrangement of their cells having become very irregular. At this stage the appearances closely resemble the general atrophy of the pancreas found in certain severe cases of diabetes in young subjects (Fig. 2).

In three weeks the atrophy of the pancreas has become much more marked. The acini are generally slightly dilated and mostly filled with clear, rather small, desquamated acinous cells. The margin of the acinus is lined by a layer of more darkly staining attenuated cells (Fig. 3). In many places the acini can be noted to have been changed into clumps of small darkly staining cells (Figs. 3 and 4).

Indeed at this stage it is almost impossible to distinguish any of the original "islands" or to differentiate from "islands" the collections of cells formed by atrophy and proliferation of the acinous cells.

In twenty-four days the lobules of atrophic acini have become isolated and surrounded by fibrous tissue. For the most part the acini are lined by an attenuated epithelium or filled with irregular or rounded desquamated and proliferated acinous cells. Many of these lobules show a more advanced atrophy than others and even in the individual clumps the atrophy is by no means regular (Fig. 5).

In thirty-seven days the atrophy is much more uniform and the pancreatic lobules have become much smaller and are surrounded by dense fibrous tissue (Fig. 6).

In thirteen weeks all that remains in the fibrous tissue which has replaced the pancreas are small groups of extremely atrophied acini. These at first sight have the appearance of Langerhans' islands, but their acinous arrangement can still be easily made out (Fig. 7). For the most part these atrophic remnants show no structures closely resembling normal "islands." In some places, however, there are found small rounded clumps of closely packed, large clear cells. These are often completely isolated in fibrous tissue but also are commonly seen in the center of a group of atrophied pancreas acini (Fig. 8). These structures might well be taken for the normal persisting "islands." They are not, however, nearly so numerous as are the normal islands in the pancreas, and the arrangement and type of their cells is also different. Further, in tracing their formation in earlier periods after ligation of the pancreatic duct, similar structures can be observed to be forming from the atrophying, catarrhal and proliferative acini. Their persistence is analogous to what is found in any injured or atrophic tissue, in which remnants are apt to survive for long periods.

After five or six months practically no further change seems to occur. Indeed it might be said that the changes after two months are comparatively slight.

In rabbits very clear pictures are obtained, as their pancreas has very large clear-celled acini and their islands are also very distinctive. After ligation of the pancreatic duct, even more rapidly than in the cat, the lobules shrink and are replaced by fibrous tissue. A week after the operation the acinous cells have become very atrophic, but catarrhal desquamation of the cells is not so marked. After ten days the "islands" can still be observed, but in many places are disappearing along with the pancreas acini.

After two months the entire separated portion of the pancreas has been replaced by fibrous tissue except for small scattered groups of very atrophic acini (Fig. 9). As in the cat, however, there are occasionally found clumps of closely packed, large clear cells of a type resembling the typical "island" without the same trabecular arrangement. Also nearly all of these groups of better preserved cells are found at the periphery of some atrophied acini which very commonly are in direct connection with them (Fig. 10).

In dogs a rather different picture is generally obtained. The tendency to reunion of the separate ends of the pancreas is remarkable. In one dog the divided ends of the pancreas had reunited so that after six weeks the point of separation could not be found and there was only a very slight degree of patchy fibrosis in the part which had been isolated. In another dog after two months the separated tail end of the pancreas did not seem to be reduced in size and had reunited with the duodenal portion. On section of this portion which had been separated off it was found to be chiefly composed of dense white fibrous tissue including numerous brownish gray soft areas an eighth to a quarter of an inch in size which closely resembled normal pancreas. These parenchymatous areas were uniformly scattered through the separated part and not only close to the duodenal attachment. On microscopic section of these parenchymatous nodules enclosed in fibrous tissue they were found to be composed of somewhat altered pancreas tissue. The acini were slightly smaller than normal. Their lumen was not dilated nor was there any

catarrh. The lining cells of the acini were much less granular and took basic stains much more deeply than the normal pancreas. Also the chromatin of their nuclei was abnormally dense. In these areas it was rare to find any definite Langerhans' islands.

Usually, however, in dogs where reunion has not occurred, the atrophy of the pancreas is very complete. After ten days the same atrophy and catarrh as in cats may be noticed, and also commencing signs of lymphocyte invasion and commencing fibrous tissue replacement. Even after three months when the separated portion of the pancreas has become only a thin fibrous strand it contains similar small nodules of only slightly altered pancreas tissue (Fig. 11). These small nodules only rarely contain structures which closely resemble "islands." Occasionally at the edge of these parenchymatous nodules there may be seen groups of small pale cells, to some extent resembling the normal "islands" of a dog, but which generally can be traced to some connection with a neighboring acinus (Fig. 12).

The morphological appearances in all these instances seem to show that following ligature of the pancreatic duct the pancreas acini rapidly become atrophied. In occasional places certain acini persist either in a well preserved state or with their cells grouped together in solid clumps. These solid clumps of cells have some resemblance to Langerhans' islands, yet are much fewer in number than the normal "islands" and the development of similar structures can be traced from atrophic catarrhal acini. As regards the fate of the "islands" they seemed to atrophy with the rest of the pancreas. Many assumed appearances indistinguishable from the surrounding atrophying acini and not infrequently presented evidences which were suggestive of an attempt to form acinous tissue.

The help that may be obtained from differential staining methods as advocated by Bensley and Lane³ in the normal pancreas, and recently affirmed by Miss Kirkbride⁴ after ligature of the pancreatic duct, is not what might be expected. This differential stain works well in the normal

guinea-pig, but at least in our experience the areas differentiated are often quite indistinguishable morphologically from ordinary acini. Sometimes, also, a whole lobule seems to take the "island" stain. In dogs the elements of the pancreas seem to stain the reverse way to guinea-pigs. In pathological processes the pancreas acini seem frequently to become modified so that their differential staining becomes very uncertain. We were unsuccessful in getting any results from differential staining of the pancreas of cats or dogs which were in any way convincing that the "islands" alone persisted after ligation of the pancreatic duct.

Indeed if the "islands" should be the only structures remaining after ligation of the pancreatic duct it would not necessarily indicate that they were the essential controlling elements of the normal pancreas in sugar metabolism. They might be portions of undifferentiated pancreas, potentially capable of development into new pancreas tissue or of performing some of the functions of the pancreas. Their independent anatomical existence, even in the normal pancreas, is questionable. Undoubtedly they exist in large numbers in the embryo and can be shown to be derived from the same source as the pancreas acini. Also by serial sections the "islands" can generally be shown to have intimate connections with the pancreatic tubules and sometimes also with the ducts. Farther even in the normal animal the "islands" are very irregular in type and apparent transitions may be observed from acini to islands and sometimes also vice versa. Hyaline degeneration occurring in the islands alone has been noted in the pancreas of some diabetic cases and been argued as a convincing proof of the functional importance and independence of the "islands." Yet we have not uncommonly observed in the pancreas of old non-diabetic subjects that the "islands" showed extensive hyaline changes. Further, similar hyaline changes are common in the pancreas acini of normal animals (Fig. 13). Sometimes also this hyaline process produces disintegration and proliferation of the acinous cells forming areas indistinguishable from islands.

In the examination of the human pancreas in diseased conditions, appearances of transition from islands to acini and vice versa are extremely common. (Some of these figures have been illustrated by one of us in the *American Journal of the Medical Sciences*, January, 1912.) It is not infrequent to find in certain old subjects that large areas of the pancreas have been entirely transformed into islands. Indeed after a study of the human pancreas in various pathological conditions the conclusion is almost convincingly suggested that the "islands" are merely resting undifferentiated pancreas tissue and that this is capable, in case of insufficiency of the organ, of changing into definite pancreas acini. This explains the common finding of completely isolated definite acinous cells in the "islands" and also how in many cases of diabetes the number of "islands" is so markedly reduced in number without leaving any scars or remnants in their places.

Perhaps the most convincing demonstration of the relative importance of the acini and "islands" of the pancreas is found in animals where pancreatectomy has been incomplete. In dogs a very minute fragment, may be no larger than a pea, if allowed to remain prevents diabetes. This fragment becomes very hypertrophic. The acini become very large and as a rule become less granular. The islands do not, however, become more numerous or larger, and in many cases no islands can be found.

In the atrophy of the pancreas which follows ligature of the pancreatic duct some of the "islands" may persist unchanged, but for the most part the "islands" atrophy with the adjacent acini or else assume appearances indistinguishable from the atrophying catarrhal acini. The great majority of the structures which persist after ligature of the pancreas are really the remnants of the atrophied acinous pancreatic tissue, and collectively represent a sufficient amount of pancreas tissue to prevent diabetes. They are cut off from any external secretion and lose their typical physical appearances, but do retain that special function of the pancreas which is indispensable to metabolism.

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4. Kirkbride. *Journ. of Exper. Med.*, 1912, xv, 101.

DESCRIPTION OF PLATES.

PLATE XVIII.

FIG. 1. — Pancreas of a cat ten days after ligature of the pancreatic duct, showing atrophy of the acinous cells.

FIG. 2. — Pancreas of a man, age thirty-four, who died from diabetes of a very rapid and severe type. In this case there is general atrophy of the pancreas. Almost no "islands" can be distinguished nor any scars or hyaline degeneration where they might have been. It closely resembles the picture shown in Fig. 1.

FIG. 3. — Pancreas of a cat seventeen days after ligature of the pancreatic duct. The acini are slightly distended and the lining cells very catarrhal.

FIG. 4. — Pancreas of a cat seventeen days after ligature of the pancreatic duct. The acini show marked catarrh. The acinous cells can be seen in certain places to be changes into clumps of small round darkly staining cells.

PLATE XIX.

FIG. 5. — Pancreas of a cat twenty-four days after ligature of the pancreatic duct. The acini for the most part have become extremely atrophied and are collected in clumps surrounded by fibrous tissue. The atrophy is not regular as numerous better preserved pancreatic acini can also be seen.

FIG. 6. — Pancreas of a cat thirty-seven days after ligature of the pancreatic duct. The acini are all extremely atrophied and collected in clumps surrounded by fibrous tissue.

FIG. 7. — Pancreas of a cat thirteen weeks after ligature of the pancreatic duct. Only small groups of attenuated acinous cells remain.

FIG. 8. — Pancreas of a cat five months after ligature of the pancreatic duct, showing clump of large, clear, closely packed cells included among atrophic pancreas acini and fibrous tissue.

PLATE XX.

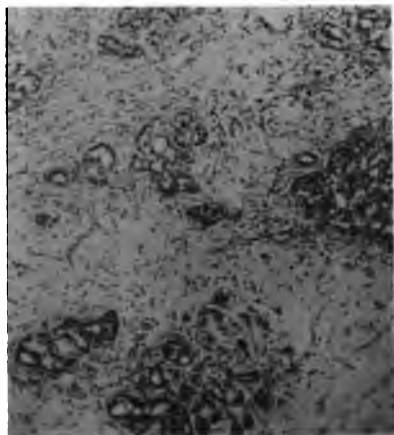
FIG. 9. — Pancreas of a rabbit nine weeks after ligature of the pancreatic duct, showing groups of acini imbedded in fibrous tissue.

FIG. 10. — Pancreas of a rabbit ten weeks after ligature of the pancreatic duct, showing inclusion of a clump of large clear cells amongst the atrophic remains of the acini. These clumps can be seen to be connected directly with the neighboring acini.

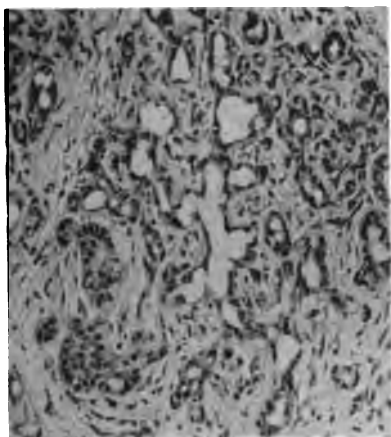
FIG. 11. — Pancreas of a dog three months after ligation of the pancreatic duct. The portion of the pancreas isolated in this case was represented by a thin strand of fibrous tissue. Included in this were several nodules of hypertrophic pancreas acini which seemed to contain no "islands."

FIG. 12. Pancreas of a dog seven weeks after ligation of the pancreatic duct showing a group of pancreas acini included in the isolated atrophic part of the pancreas. A group of clear cells not unlike a normal "island" of the dog's pancreas is seen connected with a hypertrophic acinus.

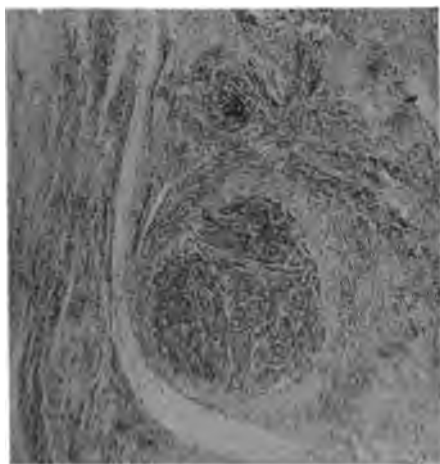
FIG. 13. — Pancreas of a normal dog showing hyaline transformation of an acinus (fixed immediately after death, in corrosive sublimate).



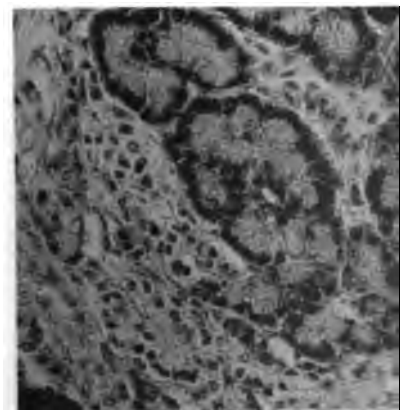
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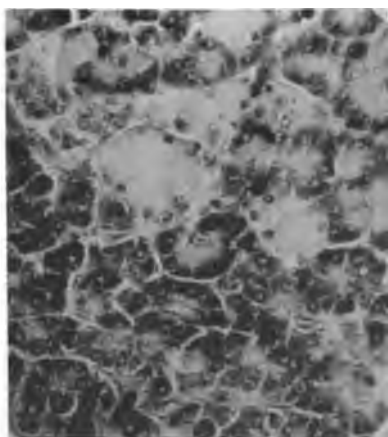
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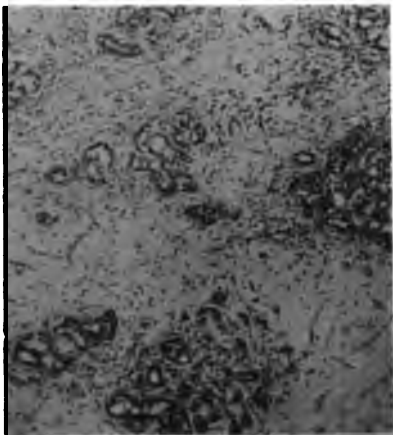
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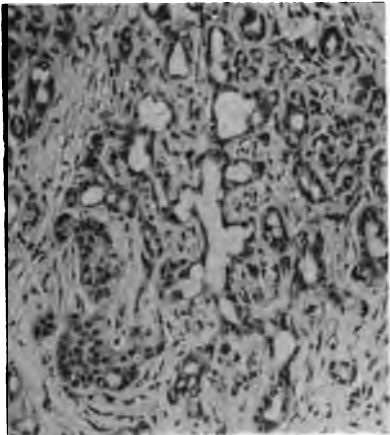
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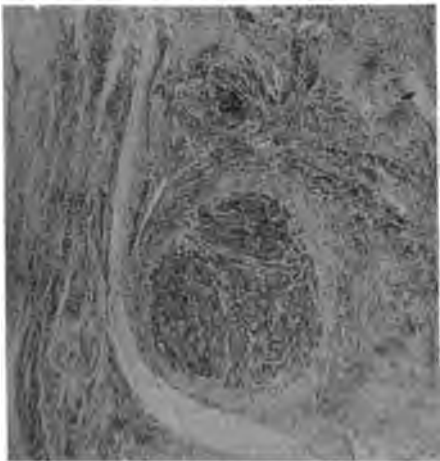
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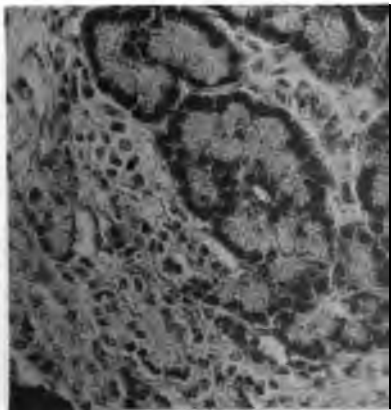
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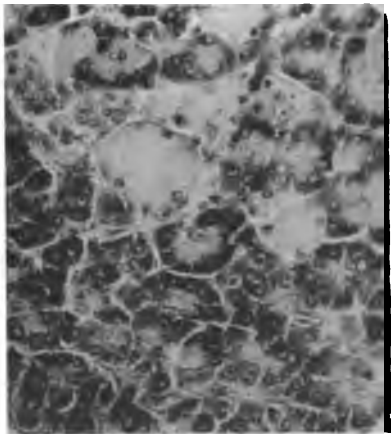
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OBSERVATIONS OF THE GLYCOLYTIC POWER OF THE BLOOD
AND TISSUES IN NORMAL AND DIABETIC CONDITIONS.*

LINDSAY S. MILNE, M.D.

AND

H. LEBARON PETERS, M.D.

(*Russell Sage Institute of Pathology, New York.*)

In numerous works particularly relating to the etiology of diabetes it has been noted that the blood possesses some glycolytic power. Indeed, one of the prevailing conceptions of diabetes is based on the failure of the normal glycolytic action. Claude Bernard was the first to consider such an idea, having observed that the sugar content of blood standing at 15° C. gradually diminished. Lépine (1890), however, was the first to attempt the exclusion of bacterial infection from his results. He considered that the destruction of glucose was brought about by a ferment which was produced in the pancreas, and in disease of that organ it was absent, thereby causing glycosuria. This, however, has been denied by Kraus, Minkowski, and others. Loewy and Richter found that hyperleucocytic bloods were more glycolytic than normal. Also it has recently been claimed by Abderhalden and Rathsmann that the normal serum of the dog possesses no glycolytic activity, but that after a large meal or the ingestion of large quantities of sugar, a marked glycolytic power is developed in the serum.

The following work has been carried out to determine if the blood possesses or can acquire any glycolytic power, and the difference in this relation between normal and diabetic animals.

Varying quantities of serum were added to sugar solutions and the sugar content of the mixture determined after different lengths of time.

The method of sugar estimation which proved most satisfactory was that described by Bertrand. This depends upon the reduction of copper

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sulphate in the presence of an alkali by glucose and the subsequent estimation of the reduced copper oxide by a ferric sulphate solution. The ferric is converted into a ferrous salt by the copper oxide, and the amount of conversion estimated by titration with a standard potassium permanganate solution. The solutions used are: (1) copper sulphate, 40 grams per 1,000 cubic centimeters; (2) Rochelle salt, 200 grams, sodium hydrate, 150 grams per 1,000 cubic centimeters; (3) ferric sulphate, 50 grams, ac. sulphuric, 200 grams per 1,000 cubic centimeters; (4) potassium permanganate, 5 grams per 1,000 cubic centimeters. The sugar solution is boiled for three minutes with a mixture of equal parts of solutions (1) and (2) and the precipitated copper oxide collected in a Gooch filter and washed. It is then dissolved in the ferric sulphate solution and the amount of conversion into ferrous sulphate estimated by titration with potassium permanganate. This corresponds to a certain amount of sugar which can be estimated by Bertrand's tables.

Dealuminizing where necessary was done by adding sodium sulphate and acetic acid in the following proportions: saturated sodium sulphate, 15 cubic centimeters; 2 per cent acetic acid, 10 cubic centimeters; serum or blood, 35 cubic centimeters; make up volume to 150 cubic centimeters with distilled water and heat slowly to boiling point. Practically the entire amount of sugar can be recovered by this method and it does not interfere in any way with the subsequent sugar estimation. Dealuminizing is, however, not absolutely necessary when Bertrand's method of sugar estimation is employed, as the albuminous precipitate does not seem to alter the amount of copper deposited. Without dealuminizing, however, filtration is often extremely slow.

The sugar was dissolved in Locke's solution (NaCl, .9 per cent; KCl, .042 per cent; CaCl_2 , .024 per cent; NaHCO_3 , .02 per cent). The same results, however, were always obtained when either normal saline (.9 per cent NaCl) or distilled water alone were used.

The blood was drawn from the femoral artery with strict aseptic precautions, local anesthesia being employed. As bacterial growth destroys sugar with great rapidity the greatest care had to be observed in the whole process of examination to avoid contamination. Indeed in those tests which became infected the small quantity of dextrose originally present had generally almost completely disappeared after twenty-four hours' incubation at 37° C.

As regards the action of blood serum on sugar, the serum of normal dogs seems to have no influence on dextrose (Table I.). The discrepancies in the results are very slight, and are probably the result of dealuminizing or in the quantitative estimation of the sugar. Five cubic centimeters of a one per cent solution of dextrose show no diminution in quantity of sugar after twenty-four hours when incubated

at 37° C. with five or one cubic centimeter of the serum of a dog fasting twenty-four hours before bleeding. This table also shows that if defibrinated blood instead of serum is used a considerable amount of dextrose is removed from the solution. In four hours a small amount of the dextrose is absorbed and in twenty-four hours as much as one-third may have been lost. After forty-eight hours a still further reduction takes place (Table II. (1)). This action, however, is prevented and the entire amount of dextrose may be recovered if the blood is previously hemolyzed by the addition of distilled water. This would seem to indicate that the dextrose is destroyed by some vital process of the blood cells and not by any action of the serum. The sugar taken up by the blood corpuscles is not merely stored up as glycogen, but to a large extent is utilized by them in their metabolism and so altered that it cannot be recovered as dextrose. This can be shown by incubating two similarly prepared blood and dextrose mixtures for twenty-four hours at 37° C. Both are then dealbuminized. If one of them is now boiled for one and one-half hours with .5 per cent hydrochloric acid, then neutralized with sodium hydrate, it is found to contain a slightly higher amount of dextrose, which is probably derived from the conversion of glycogen stored in the cells (Table I. (7), Table II. (1), Table III. (10), (11)).

As regards the possibility of the development of sugar-destroying ferments in the blood after feeding, it has been claimed by Abderhalden and Rathsmann that, after a meal, or the administration of a large quantity of sugar in dogs, the blood serum acquires marked glycolytic properties. On polariscopic examination of serum-dextrose mixtures at varying periods they observed a great reduction in the sugar content.

Table II. does not confirm this result, as far as can be determined by estimating with a copper precipitation method. Neither saccharose nor dextrose given in large quantities could be shown to produce in the serum any glycolytic activity. Similarly after a large meal there seemed to be no glycolytic agent developed in the serum (Table II. (12)).

In depancreatized diabetic dogs (Table III.), as in the normal, the serum possesses no power of destroying dextrose. It is interesting, however, inasmuch as one of the theories of diabetes depends on the idea that the cells have lost their power of taking up or using glucose, to find in dogs made diabetic either by removal of the pancreas or by the administration of phlorizin that the blood corpuscles seem to show no diminution from the normal in their ability to take up dextrose. In this series, also, the addition of water can prevent any sugar being destroyed and, as in the normal blood, only a limited amount of the sugar taken up by the blood cells can be converted back to dextrose. It may be argued that the blood cells are independent structures and do not represent the general condition of the cells of the body, but we have also found that the other tissues of diabetic dogs can take up dextrose as in the normal.

Table IV. indicates the result of perfusion of sugar solution through the liver and limbs of normal and depancreatized diabetic dogs.

The perfusion fluid was composed of dextrose one per cent in Locke's solution and was passed through at 37° C. The limb or liver of the animal was removed from the body after the animal had been killed by bleeding. The limb was first perfused with one thousand cubic centimeters Locke's solution, then with two hundred and fifty cubic centimeters Locke's solution containing one per cent dextrose. After this five hundred to eight hundred cubic centimeters of oxygenated dextrose solution were circulated through the limb for one hour. From this third perfusion the loss of sugar noted in Table IV. was estimated. As an average, eight hundred cubic centimeters perfused through a limb six to eight times in an hour and through the liver twelve to fifteen times per hour. Estimations were made from the perfusion fluid at quarter-hour intervals.

In the initial muscle perfusion with Locke's solution practically no sugar could be found, but in the liver there was quite an appreciable trace. In the course of the perfusion the estimations at various intervals before the final result

showed a gradual decline in the sugar content of the perfusion fluid indicating the continued absorption of a considerable amount of dextrose. Perfusion of the liver, however, in several instances showed a slight gain in the dextrose content. This was noticed particularly where the temperature of the fluid perfusing the liver was much above or below 37° C. (Table IV. (12)).

Depancreatized diabetic dogs gave a similar or even more marked result which indicates that the tissues as well as the blood corpuscles of the diabetic cannot only take up dextrose but may have even a greater than normal power in this direction.

It has been claimed that the muscles possess very little power of taking up dextrose except when influenced by some activating substance derived from the pancreas, yet the isolated organs of the diabetic as in the normal can absorb a considerable quantity of glucose. Also, as is shown in Table IV. (3) and (4), the perfusion of a pancreas in the same circuit as a limb makes very little difference in the amount of sugar removed from the solution.

If the blood serum can be shown to have no apparent action on dextrose its results on glycogen are very different. The serum seems to contain some substance which has the power of converting glycogen into dextrose. In the dog this action is particularly marked and is eight to ten times stronger than in human serum. This substance in the serum has the characteristics of an enzyme, a small amount eventually completely converting a relatively large quantity of glycogen. The rate of conversion of a given amount of glycogen, however, depends on the amount of serum. Table V. shows that this enzyme can produce dextrose from glycogen in very definite proportions. For instance ten cubic centimeters of one per cent glycogen is almost completely converted into dextrose by five cubic centimeters dog's serum in four hours at 37° C., whereas one-half cubic centimeter serum has been able to convert only a very small proportion of the glycogen. Only after three days with one-half cubic centimeter serum does the amount converted

approach that produced by five cubic centimeters serum in four hours.

This glycogen converting enzyme has certain definite properties. It is comparatively stable and does not markedly lose its power until twenty-four hours after the blood is drawn. It may retain its activity unimpaired for a considerably longer period if the serum is kept in a refrigerator. The optimum temperature for its activity is about 40° C. Its action is reduced by about half at 15° C. and arrested almost completely at 0° C. Dog serum incubated at 56° C. for half an hour loses all its property of glycogen conversion. This diastatic action occurs equally well if the glycogen is dissolved in distilled water, in .9 per cent sodium chloride, or in Locke's solution, and in any degree of dilution. The amount of glycogen in the solution, however, does alter the result. In a two per cent solution of glycogen there is generally considerably more conversion than in one per cent.

Defibrinated blood converts in proportion to the serum contained in it, some of the dextrose produced also being taken up by the blood corpuscles.

In comparative estimations, then, of the diastatic action of serum on glycogen, the temperature, the time of incubation, the amount of serum and glycogen, and the presence of bacterial infection have to be carefully noted. Also the dogs examined must all be about the same size and age.

Table VI. shows the action of normal dog's serum on glycogen. Dogs of a certain size give fairly uniform results. Large dogs (No. 9 and No. 10), however, generally give slightly higher figures. It can also be seen from this table that in fasting the diastatic action of the serum is not lost, and may indeed appear to be slightly increased (Table VI. (12), (13)). This latter condition may be due, however, to the fact that in fasting the serum becomes extremely concentrated, and therefore the amount of enzyme per volume might be increased. The ingestion of a large meat or carbohydrate meal or a large amount of glucose does not seem to increase the power of the serum to convert glycogen (Table

VI. (14), (15), (16), (17)). This enzyme is not then any immediate result of the ingestion of food.

The comparison of these results with what is found in diabetes is of interest, as it might well be that the excessive amount of sugar in the blood is produced by an increased diastatic action of the serum causing an excessive conversion of glycogen into glucose. To determine this a series of dogs of about the same size as used for estimation of the diastatic value of the normal serum were depancreatized, and in this relation it may be noted that the persistence of a small pancreatic fragment may prevent diabetes. Also even if severe glycosuria does result after the operation, recovery is not uncommon and the autopsy in such cases reveals a small nodule of hypertrophic pancreas tissue, maybe no larger than a pea.

In depancreatized diabetic dogs the diastatic power of the serum tends to become slightly increased (Table VII.). It could also be noted that in those dogs where glycosuria stopped, the diastatic power of the serum became much lower than during the diabetic period (Table VII. (12), (13), (14)). In no case, however, where either partial or complete pancreatectomy was performed was the diastatic action of the serum diminished. In this respect it varied from the results reported by Gould and Carlson on starch. They found that the diastases in the blood serum affecting starch gained markedly in power after ligature of the pancreatic duct, particularly forty to seventy-two hours after the operation, with a gradual return to normal, but after pancreatectomy became much reduced in strength, and only after some time approximated again to the normal.

It may be that this increase of power to convert glycogen in diabetes is too small to be of importance, and is the result of lessened alkalinity, or of concentration of the serum. In fasting, however, there may be much greater concentration of the serum than in diabetes, yet the diastatic action is not so powerful.

If now the tissues in pancreatic diabetes can utilize dextrose, and since their glycogen content is usually reduced,

the disease would seem to depend on an excessive production of glucose from stored glycogen. This increased production may be the result of the failure of secretion of an antidiastatic enzyme or, as seems more probable, due to the action of accumulated substances in the serum which normally should be destroyed or altered by the pancreas. These substances increase slightly the diastatic power of the serum, but their chief action more likely is in the direction of causing the tissue cells to convert their store of glycogen into glucose. As an analogous process it is not unlike what in certain conditions occurs in the fat metabolism of cells.

When the amount of glycogen stored in the tissues is reduced by starvation then the sugar excretion is naturally diminished. Marcuse has shown that in frogs the bulk of the glycogen of the body is stored in the liver, and after the liver is removed pancreatectomy fails to produce diabetes.

In phloridzin diabetic dogs the results are not quite similar, as there does not seem to be any special increase of diastase in the serum (Table VIII.). In this series large dogs were used. They were first bled, then injected with phloridzin and two to four hours later when glycosuria had become evident they were again bled. Several dogs were kept phloridzined for several days, but as long as there was glycosuria the results were practically the same as in those examined earlier. In only one dog (Table VIII. (5)) was there any definite diastase increase. In Number 7 a slight increase may be noticed, due, however, to the increased amount of sugar in the serum. Very commonly the amount of sugar produced from glycogen was slightly less in the second bleeding, four hours after injection, owing probably to dilution of the serum. It was uniformly found that after the glycosuria had passed off, the power of the serum to convert glycogen was considerably lowered, just as in those depancreatized diabetic dogs when glycosuria stopped.

In every case, also, while there was glycosuria, the amount of sugar in the blood serum appeared to be slightly increased. In some cases (Nos. 1, 7, 8) the amount of sugar in the

serum had more than doubled. In cases Nos. 2, 3, 4, 6, and 9 the increase was only very slight. This increased sugar in the serum in phloridzin diabetes has also been reported by Pavy, Leone, Coolen, and Bidel and Kolisch, but a hypoglycemia as noted by Minkowski and von Meh-ring has been practically uniformly described. It may be doubted if this slight increase of sugar in the serum is alone responsible for the glycosuria, yet it renders the explanation of phloridzin diabetes on a purely renal basis more difficult to understand, even although one agrees that an increased quantity of sugar may be passed into the serum to meet a demand on the part of the tissues started by the drainage of sugar from the blood through the kidneys. In this relation it has to be noted that only very slight increase in the sugar content of the serum may be associated with glycosuria. Thus in feeding dogs with dextrose, glycosuria (.6 per cent) may exist with a serum containing .18 per cent sugar. Indeed a concentration of over .1 per cent sugar in the serum is apt to be associated with glycosuria.

Phloridzin by itself in alkaline or in alcoholic solution has no action on glycogen. If phloridzin in alkaline (sodium carbonate one per cent) or alcoholic solution be added to a mixture of serum and glycogen, then there is rather less dextrose converted than the corresponding amount of serum alone could produce.

Although phloridzin itself does not change glycogen nor increase the diastatic action of the serum, yet there are numerous observations which indicate that phloridzin, just as pancreatic diabetes, is associated with an increased production of sugar by the tissues. It has been shown by Pflüger and Junkersdorf that glycogen disappears from the organs in proportion to the time after administration of phloridzin and is again rapidly restored to its normal amount after the glycosuria stops. Fischera denied these results, but on grounds which have been criticised by Bleibtreu and Kato. Grube perfused the livers of tortoises, one side with a dextrose solution and the other with a dextrose solution containing phloridzin. On the phloridzin side there was considerably

less glycogen and he concluded that phloridzin caused the tissues to lose their power of forming glycogen. The same result was obtained using Ringer's solution in place of dextrose. Suckrow got much the same results, but in five out of fourteen of his cases there was more glycogen in the side of the liver exposed to phloridzin. He also showed the uncertainty of such results, as he found that the normal glycogen content of the two sides of the liver may vary as much as 15.4 per cent. Levene found that, after injection of phloridzin solution into the kidney the sugar content of the organ is increased and that there is slightly more sugar in the renal vein than the renal artery, a difficult fact to explain on the theory that phloridzin diabetes was simply a filtration through the kidney. Cornevin also noted that the milk in phloridzin diabetes contains an increased amount of sugar. Again it has been noticed that in fasting where the glycogen content of the organ is much reduced, larger doses of phloridzin are necessary to produce glycosuria.

It seems possible that phloridzin acts in the direction of causing the tissue cells to form dextrose from the glycogen stored in them. The slight increase in the amount of serum sugar may be and probably is sufficient to cause the glycosuria. It is difficult, however, to disprove any change in the epithelium of the excretory organs and it is also possible that the glucose produced from glycogen in the tissues is in a condition specially easily eliminated.

In ether anesthesia a condition is produced which is closely related to phloridzin diabetes. After ether anesthesia it has been observed by King, Hawk, Selig, McLoed, and others, that glycosuria is common. These observers also noted that the sugar content of the blood is increased, but generally only slightly. Indeed Hawk doubted that it was sufficient to cause glycosuria. He considered, however, that the hyperglycemia was the result of an increased stimulation of the production of glucose from glycogen. McLoed and Pearce also showed in such cases, as in phloridzin diabetes, that the glycogen of the organs becomes considerably

diminished. In King's experiments the increase of the sugar content of the blood in cases of glycosuria after ether anesthesia varied between .049 per cent and .184 per cent. Sugar was found in the urine with only .111 per cent sugar in the serum.

Sugar puncture of the medulla oblongata has been noticed to be followed by a disappearance of glycogen from the liver. Possibly, however, this is produced by vasomotor changes causing pancreatic insufficiency, as it is known that even slight alterations of the vascular supply of the pancreas may completely arrest its action. Adrenalin painted on the pancreas is followed by glycosuria to a greater degree than when it is administered in any other way. Further, in fasting, where the glycogen of the organs is much reduced, puncture of the medulla may produce no glycosuria.

In all forms of diabetes, then, either as the result of pancreatectomy, ether, sugar puncture of the brain, or phloridzin, there is a rapid diminution of glycogen in the tissues. This is associated with an increased formation of glucose, at times considerable, and which results in hyperglycemia and glycosuria. The ability to form glycogen, however, still remains, so that new glycogen can continually be produced.

This increased conversion of glycogen into glucose may be considered as due to the direct action of certain substances, such as phloridzin, ether, or products retained in the serum by pancreatic insufficiency, which increase the diastatic power of the blood serum, or act on the tissue cells so that they rapidly convert their stored glycogen into glucose.

CONCLUSIONS.

1. Normal dog serum has no glycolytic action on glucose.
2. After a large meal or the administration of a large quantity of dextrose or saccharose the serum acquires no glycolytic power.
3. Blood corpuscles can absorb a comparatively large amount of glucose.

4. Only a small amount of this glucose absorbed by blood corpuscles can be recovered as dextrose.

5. The blood of depancreatized dogs has a normal or slightly increased power of taking up dextrose and only a small amount of this can be recovered as dextrose.

6. The tissues of depancreatized diabetic dogs can absorb as much as normal, or more, dextrose from a perfusion fluid.

7. Normal dog serum has a marked diastatic action on glycogen, converting it into glucose. Its strength is not altered after a large meal or administration of large quantities of dextrose, in fasting, or in phloridzin diabetes, but is slightly, often markedly, increased in depancreatized diabetic dogs.

8. In phloridzin diabetes the sugar content of the serum is slightly increased, often in sufficient degree to easily account for its elimination in the urine.

TABLE I.
Normal dogs (no food for twenty-four hours before bleeding). (Results in milligrams of glucose.)

	Glucose Solution 5 cc. plus 5 cc. Serum, 24 Hours, 37°.	Glucose Solution 5 cc. plus 1 cc. Serum, 24 Hours, 37°.	Glucose Solution 5 cc. plus 5 cc. Blood, 24 Hours, 37°.	Glucose Solution 5 cc. plus 100 cc. Water, 24 Hours, 37°.	Glucose Solution 5 cc. plus 5 cc. Blood, 24 Hours. (Filtrate Boiled 1 Hour with 1% HCl. Neut. with NaOH.)	Glucose Solution, 5 cc.	Serum, 5 cc.
1	52.6	50.2	38.5	51.6	4.9
2	54	(1) 35.6 (2) 38.5	52.1	3.6
3	86	76.5	82.1	4.2
4	83.2	69	80	3.9
5	58.2	52.9	52	5.9
6	61.6	(1) 53 (2) 53.6	62.2	58.5	9.2 (blood 5.3)
7	43	28.6	31.4	39.9	3.6 (blood 1.9)

TABLE II.
Dogs fed with saccharose or dextrose. (Results in milligrams of glucose.)

	Glucose Solution 5 cc. plus 5 cc. Serum, 24 Hours, 37°.	Glucose Solution 5 cc. plus 1 cc. Serum, 24 Hours, 37°.	Glucose Solution 5 cc. plus 5 cc. Blood, 24 Hours, 37°.	Glucose Solution 5 cc. plus 5 cc. Blood plus 100 cc. Water, 24 Hours, 37°.	Glucose Solution 5 cc. plus 5 cc. Blood (Filtrate Boiled 1 Hour with 1% HCl, Neutralized with NaOH.)	Glucose Solution, 5 cc.	Serum, 5 cc.
1. Saccharose, 250 grams 4 hours before bleeding (trace of glucose in urine)	48.6	46.2	33 (after 48 hours, 28.1)	37.3	46.5	3
2. Saccharose, 150 grams 4 hours before bleeding	61.9	59.4	58.1	6.3
3. Saccharose, 150 grams 3 hours before bleeding	59.8	43.05	54.2	55	4.3
4. Dextrose, 150 grams 4 hours before bleeding	61.2	58.8	58.1	4.8
5. Dextrose, 150 grams 3 hours before bleeding (sugar in urine)	54.5	46.8	43.3	11.7
6. Dextrose, 150 grams 4 hours before bleeding (sugar in urine)	55.9	53.5	13.5
7. Dextrose, 150 grams 2 hours before bleeding	86.5	85	76.1	84	4.8

8. Dextrose, 100 grams with large carbohydrate meal 3 hours previously.....	46.1	44.3	43.7	7.2
9. Dextrose, 100 grams with large carbohydrate meal 3½ hours previously.....	47.2	42	43.7	5.7
10. Dextrose, 100 grams 3 hours before bleeding (dog with greater part of pancreas removed — not diabetic).....	58.2	56.5	7.9
11. Dextrose, 60 grams daily for 8 days, bled 3 hours after last feeding. Very small amount of serum blood.....	50	47.8	40.5	47.3	2.9
12. Large meal of bread and meat 3 hours previous to bleeding.....	53.1	44.6	53.05	2.9
13. Starvation 8 days (very small amount of serum in blood).....	53.4	53.05	2.8
14. Phloridzin diabetic dog.....	55.5	32.7	51.9	52.3	3.2

TABLE III.
Depauperized diabetic dogs. (Results in milligrams of glucose.)

	Glucose Solution 5 cc. plus 5 cc. Serum, 24 Hours, 37°.	Glucose Solution 5 cc. plus 1 cc. Serum, 24 Hours, 37°.	Glucose Solution 5 cc. plus 5 cc. Blood, 24 Hours, 37°.	Glucose Solution 5 cc. plus 5 cc. Blood plus 100 cc. Water, 24 Hours, 37°.	Glucose Solution 5 cc. plus 5 cc. Blood, 24 Hours, 37°. with 14 HCl. Neut. with NaOH.)	Glucose Solu- tion, 5 cc.	Serum, 5 cc.
1.....	74.5	67	64.5	11.5
2.....	58.8	48.3	9.5
3.....	73.5	67.6	66.5	10.5
4.....	64.5	54.5	49.5	16.1
5.....	63.3	54.5	48.3	12.9
6.....	65.2	55.8	48.9	16.1
7.....	65.5	(1) 33.8 (2) 32.1	49.5	20.4 (blood 9.5)
8.....	69.7	42.5	58	53.1	21 (blood 10)
9.....	80.1	58.5	55.1	27 (blood 18.5)
10.....	59.6	32 (60 hours, 26)	33.7	39.9	22 (blood 6.9)
11.....	57.1	18.8	21.7	39.9	19.2 (blood 5.8)

TABLE IV.
Perfusion of tissues with glucose solution.

Animal.	Tissue Perfused.	Amount Perfused.	Duration of Perfusion.	Titer of Solution after First Perfusion.		Titer after Final Perfusion.		Estimated Amount Lost per 100 cc.		Total Amount Lost.	
				Dextrose, 1%.		Milligrams per 100 cc.		Milligrams per 100 cc.		Milligrams.	
1. Normal dog	Leg.	800 cc.	1½ hours.	800 cc.		912		826		86	
2. "	"	800 "	1 hour.	800 "		900		832		78	
3. "	Left leg and pancreas.	500 "	1½ hours.	500 "		696		606		90	
4. Same animal as No. 3.	Right leg.	875 "	1½ "	875 "		900		854		46	
5. Diabetic dog.	Leg.	550 "	1 hour.	550 "		976		790		186	
6. "	"	800 "	15 minutes.	800 "		952		924		28	
7. "	"	800 "	1 hour.	800 "		848		734		114	
8. "	"	900 "	45 minutes.	900 "		714		664		50	
9. Recovered, diabetic dog	"	750 "	1 hour.	750 "		700		530		170	
										1275	

TABLE IV. — *Continued.*

Animal.	Tissue Perfused.	Amount Perfused.	Duration of Perfusion.	Titer of Solution after First Perfusion.	Titer after Final Perfusion.	Estimated Amount Lost per 100 cc.	Total Amount Lost.
		Dextrose, 1%. cc.		Milligrams per 100 cc.	Milligrams per 100 cc.	Milligrams.	
10. Dog, same as No. 1...	Liver.	850 cc.	1½ hours.	958	910	48	408
11. Diabetic dog (same as No. 7).....	"	800 "	1 hour.	916	836	80	640
12. Diabetic dog (same as No. 8).....	"	800 "	1 "	880	976	96 (gained)	768 (gained)
13. Recovered, diabetic dog (same as No. 9)	"	700 "	1 "	876	698	178	1246

TABLE V.

(Results in milligrams of glucose.)

Normal dog serum, 1/20 cc., + Glycogen, 10 cc. (2 per cent), 4 hours at 37°.											3.5
"	"	"	1/10	"	+	"	10	"	(2	"), " " " "	9.5
"	"	"	1/5	"	+	"	10	"	(2	"), " " " "	14.5
"	"	"	1/2	"	+	"	10	"	(2	"), " " " "	22.2
"	"	"	1	"	+	"	10	"	(2	"), " " " "	28.8
"	"	"	3	"	+	"	10	"	(2	"), " " " "	38.5
"	"	"	5	"	+	"	10	"	(2	"), " " " "	50.1
"	"	"	10	"	+	"	10	"	(2	"), " " " "	60.7
"	"	"	10	"							10.2

TABLE VI.
Normal dogs. (Results in milligrams of glucose.)

	Glycogen 10 cc. 1½ plus 5 cc. Serum, 4 Hours, 37°.	Glycogen 10 cc. 1½ plus 5 cc. Serum, 24 Hours, 37°.	Glycogen 10 cc. 1½ plus 5 cc. Serum, 4 Hours, 37°.	Glycogen 10 cc. 1½ plus 5 cc. Serum, 24 Hours, 37°.	Serum, 5 cc.
1. Terrier	33.2	50.2	11.5	28.6	4.9
2. "	35.3	51.2	13.6	21.2	6.9
3. "	39.1	48.5	14.6	25.6	4.3
4. "	48.2	15.8	25.1	9.2
5. "	41.3	15.1	28	5
6. "	29.2	13.9	24.9	3.6
7. "	34.1	10.9	19.6	2.1
8. "	32.3	13.9	23.5	1.6
9. "	33.9	14.9	4.5
10. Large collie	49	49.5	13.9	26.9	4
11. Large bull terrier	49.4	15.1	38.6	3.5

12. Fasted 8 days — terrier (very small proportion of serum in blood)	48.8	12	29.5	2.9
13. Fasted 8 days — terrier (very small proportion of serum in blood)	45.1	12.5	21.2	2.9
14. Meat fed 8 days. Last fed 3 hours before bleeding — terrier	34.6	14.6	26.8	2.4
15. Dextrose 150 grams 3 hours before bleeding. 4% glycosuria (same as No. 7)	43.9	65.8	16.9	41	11.7
16. Dextrose 100 grams and large carbohydrate meal 3 hours previously (same as No. 11)	49.5	61.5	15.5	32.9	7.2
17. Dextrose 100 grams and large carbohydrate meal 3½ hours previously (same as No. 8)	34.6	43.1	10.2	21.8	5.7

TABLE VII.
Depancreatized diabetic dogs. (Results in milligrams of glucose.)

Time after Operation.	Sugar in Urine for 24 Hours before Bleeding.	Glycogen 10 cc. 1% plus 5 cc. Serum, 4 Hours, 37°.	Glycogen 10 cc. 1% plus 5 cc. Serum, 24 Hours, 37°.	Glycogen 10 cc. 1% plus 4 cc. Serum, 4 Hours, 37°.	Glycogen 10 cc. 1% plus 4 cc. Serum, 24 Hours, 37°.	Serum, 5 cc.
1. 3 days.....	6%	92.4	118	35.9	58.2	13.2
2. 4 days.....	9.7%	24.1	57.2	13.3
3. 8 days (same animal as No. 2).....	7%	66.8	120.1	18.7	40.4	9.5
4. 4 days.....	4%	53.9	19	30.9	16.1
5. 7 days (same animal as No. 4).....	2%	49.6	65.8	17.2	28	12.9
6. 6 days.....	5%	57.6	120	16.1	27.8	16.1
7. 9 days (same animal as No. 6).....	11.2%	67	16.9	28.6	20.2
8. 3 days.....	4.2%	71.5	28.4	20.4
9. 4 days.....	5.4%	61	19.8	33.7	21.2
10. 4 weeks.....	6.2%	69.7	82	16.8	29	17
11. 5 days.....	8%	67	21.05	36.2	10.5
12. 2 days later (same animal as No. 11).....	Only faint trace of sugar. None next day.	33.2	9	26.2	2.5
13. 8 days later (same animal as No. 10).....	No sugar in urine.	39.5	52.6	10.9	3.5
14. 7 weeks.....	3.1 to 4.6% sugar in urine for first 8 days — none since.	29.1	8.3	23.1	7.8

TABLE VIII.
Phloridized dogs. (Results in milligrams of glucose.)

	Glycogen 10 cc. 1½ plus 5 cc. Serum, 4 Hours, 37°.	Glycogen 10 cc. 1½ plus 5 cc. Serum, 24 Hours, 37°.	Glycogen 10 cc. 1½ plus 5 cc. Serum, 4 Hours, 37°.	Glycogen 10 cc. 1½ plus 5 cc. Serum, 24 Hours, 37°.	Serum, 5 cc.
1. Normal	45.2	60.8	15.4	28.6	2.5
Same animal 3 hours after injection with 2 grams phloridzin. Glycosuria before bleeding	46.4	59.9	14.6	30.2	5.6
2. Normal	29.2	13.9	24.9	3.6
Same animal 2 hours after injection with 2 grams phloridzin. Glycosuria before bleeding	39.5	13	23.6	4.1
3. Normal	34.1	10.9	19.8	2.1
Same animal 2 hours after injection with 2 grams phloridzin. Glycosuria before bleeding	28.6	10.9	17.5	2.9
4. Normal	37.2	16.5	29.7	5.2
Same animal 2 hours after injection of 3 grams phloridzin. Glycosuria before bleeding	37.8	14.7	25.6	6.5

TABLE VIII. — *Continued.*

	Glycogen 10 cc. 1½ plus ½ cc. Serum, 4 Hours, 37°.	Glycogen 10 cc. 1½ plus ½ cc. Serum, 24 Hours, 37°.	Glycogen 10 cc. 1½ plus ½ cc. Serum, 4 Hours, 37°.	Glycogen 10 cc. 1½ plus ½ cc. Serum, 24 Hours, 37°.	Serum, 5 cc.
5. Normal	41.2	15.1	38	3
Same animal 3 hours after injection of 2 grams phloridzin.	53.4	19.6	45.2	4.4
Same animal 36 hours later. No glycosuria now.....	35.8	10.4	1.6
6. Normal	49.4	15.1	38.6	3.5
Same animal 3 hours after injection of 3 grams phloridzin.	47.4	15.2	38	4.6
7. Normal (large collie)	49	13.9	26.9	4
Same dog 3 hours later after phloridzin 4 grams. (Glycosuria marked)	54.5	17.6	37.4	9.05
8. Normal (bull dog).....	57.9	23.2	49	3.1
Same dog 3 hours later after 3 grams phloridzin.....	47.8	21.3	49.5	8.8
9. Normal (bull terrier).....	55	15.9	38.8	3
3 hours after 2 grams phloridzin	53.8	16.3	42.2	3.6

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A CONTRIBUTION TO THE PATHOGENESIS OF B. ABORTUS, BANG. — II.*

MARSHAL FABYAN, M.D.

(From the Laboratory of Comparative Pathology, Harvard University Medical School, Boston, Mass.)

HISTORICAL REVIEW.

That abortion in cattle was contagious in many instances, and might spread from animal to animal in a herd, was known to farmers and breeders in the early part of the nineteenth century. The veterinarians did not, however, at first accept this view. It was considered by many the most fatal disease, after tuberculosis, to which cattle were susceptible.

In 1826 Hutrel d'Arboval, a Frenchman, and in 1834 Youatt, an Englishman,¹ came to the conclusion that abortion often was contagious and not due to environment or accidents. Their work was confirmed by many investigators.

In 1878 Lehnert² was able to demonstrate this contagiousness by experiment. He caused abortion by introducing into the vagina of pregnant cows the vaginal discharge and placental tissue of aborted cases. In 1880 this was confirmed by Brauer³ and others.

In 1886 Nocard⁴ made extensive bacteriological investigations, studying the fetus and membranes of abortion cases in the hope of isolating the definite etiological factor. He was able to obtain a bacillus and a micrococcus in pure culture, but in neither of these was he able to produce abortion.

In 1894 Sand⁵ also asserted the infectious character of the disease and gave various clinical data.

In 1895 Bang and Stribolt⁶ were able to demonstrate the at present accepted etiological organism. They obtained a cow with all the symptoms of impending abortion and, having slaughtered her, removed the unopened uterus to the laboratory. This was opened with all aseptic precautions

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and an abundant yellowish, odorless exudate was found between the uterine mucosa and the chorion. This exudate contained numerous very small bacilli, apparently in pure culture. These were found singly, and in clumps; free, and often intracellular.

They were able to grow this organism in a solid medium composed of equal parts of .75 per cent agar, five per cent gelatine and sterile serum. The last was added after the agar had been liquefied and cooled to 45° C. The still liquid medium was then inoculated with the material, gently agitated, and placed in cold water to allow rapid solidification (this medium will be referred to as A. G. S.). At the end of two to four days' incubation at 37° C. they found that a zone of growth had developed, composed of very small colonies, the largest the size of a pinhead. This zone lay one-half centimeter below the surface of the medium and was of a thickness of one to one and one-half centimeters. If inoculated tubes, similar to the above, were placed in an atmosphere of oxygen, two zones of growth developed, one near the surface of the medium, the other close to the bottom. The bacillus might also be made to grow on the slanted surface by the introduction of oxygen. It would not develop in the ordinary atmosphere nor in the absence of oxygen as produced with alkaline pyrogallol. No growth followed incubating under the influence of carbon dioxide or nitrogen. Exhausting the air over the medium caused extension of the growth to the surface and this method was employed in making plate cultures but was not reliable. The organism might grow in serum glycerine bouillon. They concluded that the bacillus was neither an aërobe nor an anaërobe, but lay in an intermediate group requiring a pressure of oxygen less than air. By their method they were able to isolate *B. abortus* in a number of cases from the placenta. Abortion was produced with these isolated cultures by injecting healthy pregnant animals in various ways. The intravenous route was apparently the most certain.

In 1901 Preisz⁷ at Budapest isolated a similar organism from the vaginal discharge of a case of abortion. He made

his inoculations directly on slanted ordinary agar made of meat infusion, peptone and salt, and after passing oxygen into these tubes he sealed them with wax and obtained a growth visible with a hand lens in three days. Inoculated into deep dextrose agar, the zonal growth appeared lying seven to fifteen millimeters beneath the surface of the medium. Stab cultures of the organism rarely reached the surface after prolonged incubation. He considered that his medium was as favorable as the A. G. S. of Bang. He was able, by using alkaline pyrogallol, to produce a growth and also by using acetylene gas. He believed that in these two instances growth resulted because oxygen was not wholly absent. He compared the organism to an anaërobe, but *B. abortus* differed in that it would also grow in pure oxygen. Small pregnant animals were injected according to Bang's method, but without positive results, and he concluded that his inoculations were made too early or his cultures had been grown too long artificially. He made note of the fact that no local lesion occurred from subcutaneous inoculation.

In 1908 Nowak,⁸ at the University of Krakau in Austria, took up the study of this organism, being attracted by its very interesting biological characteristics. He stated that its behavior towards oxygen was not without example, as was demonstrated by the work of Righi and Beijerinck. In studying pure material he found the method of Bang satisfactory, but where contaminations were present the growth might easily be killed out or masked. The broad surface which Petri dishes afforded was desirable to obtain isolated colonies. Preisz, by the use of alkaline pyrogallol, had been able to obtain growth on plates, but it was impossible to tell how much absorption had occurred and his results were uncertain. Nowak therefore sought to obtain an oxygen tension less than air by the use of a closed chamber containing the actively growing culture of an organism like *B. subtilis*, a method which had already been employed in the removal of oxygen in tetanus cultures. He placed tubes inoculated, some with *B. subtilis* and others with *B. abortus*, in a glass chamber closed with paraffine and placed this in

the incubator. Satisfactory results were obtained when the relation of the surface of *B. subtilis* was in proper proportion to the volume of the chamber. If too little of *B. subtilis* was present, no growth resulted; if too much, all the oxygen was absorbed and *B. abortus* checked. He found one square centimeter of surface growth of *B. subtilis* to fifteen cubic centimeters of volume the best proportion. Different varieties of *B. subtilis* gave similar results. The tubes inoculated with suspected abortion material were first incubated twenty-four hours in the large chamber of the incubator in order to develop any contaminations, and then incubated under the influence of *B. subtilis*, when typical colonies developed in the clear areas. He tested the purity of his cultures by the zonal growth of Bang. By gradually using less of *B. subtilis*, he trained *B. abortus* to grow in a normal atmosphere. In pure oxygen two out of six cultures grew. Under compression he obtained good results from three atmospheres but no growth at six atmospheres, although when this latter tube was placed under the influence of *B. subtilis* colonies rapidly developed. By his technic he was able to obtain many cultures from the fetus and vaginal discharge where other methods failed. He experimented only on small animals, and produced abortion with his cultures by the subcutaneous, intravenous, and intra-abdominal routes, but not by the vagina or by feeding.

In 1909 McFadyean and Stockman investigated contagious abortion in Great Britain and were able to isolate an organism similar to the one obtained by Bang in Denmark. They worked almost exclusively with the A. G. S. medium, but observed such departures from the ordinary cultural characteristics of *B. abortus* that they were obliged to test their cultures on animals to be sure that they were dealing with the same organism. Even the sub-surface growth in deep medium described by Bang as specific for *B. abortus* could not be relied on, for in one instance the tubercle bacillus grew in a like manner.

They considered *B. abortus* a strict aërobe, demonstrating that anaërobic conditions proved fatal to it. The organism

might develop in an atmosphere of coal gas, but this was explained by the fact that all the oxygen was not replaced. That the *B. abortus* does absorb oxygen was evident from the lessened internal pressure in the inoculated sealed flasks after prolonged incubation and this might destroy the flask. They, as well as Nowak, appreciated the fact that many of their tubes remained sterile although known to be inoculated with *B. abortus*. At times only a few of many tubes inoculated and incubated alike developed colonies. They produced abortion experimentally by injecting the exudate from cases studied and also the cultures isolated. The latter caused abortion more constantly. The injections were made subcutaneously and intravenously as well as by mouth and vagina. It is an interesting fact, in view of our work, that they inoculated each of two guinea-pigs with two cubic centimeters of emulsion of an exudate, one into the peritoneum, the other subcutaneously. The exudate was known to contain *B. abortus* both from cultural tests and further animal experimentation. However, "both guinea-pigs remained well for two months when they were killed and examined. No lesions of any kind were found." They therefore concluded that the exudate contained nothing pathogenic for non-pregnant guinea-pigs.

In 1910 Zwick¹⁰ issued a preliminary report on the bacteriological investigation of contagious abortion made by the German Imperial Health Office. It was demonstrated that the Bang bacillus was the etiological factor in causing this disease in England, Germany, Holland, and Denmark. A series of cultures showed individual differences among different strains, but the organism could be grown readily on various ordinary laboratory media and they confirmed Nowak's observation that the organism could be made to grow readily in the ordinary atmosphere. In fact, in one of their cases the organism was grown aërobically from the animal body. They have met with success in causing abortion by inoculations.

In this country the disease has been known for some time

and has been studied in the Agricultural Experiment Stations of Kansas, Arizona, Connecticut, Illinois, and Wisconsin. Investigators in America had been unable to confirm Bang's researches, and in 1910 MacNeal and Kerr¹¹ studied eighteen cases, ten normal deliveries, and eight premature, of which six might be called, clinically, contagious abortion. Of these, two were cultured by Nowak's method with positive results.

More recently Larson¹² confirmed the presence of *B. abortus*, Bang, in this country, by complement-deviation tests made at the Wisconsin Agricultural Experiment Station. Five herds showed the presence of this organism. (Within a few weeks Larson has reported some interesting results from applying the complement-deviation tests to human serum.)

In 1911 Holth¹ described his method for isolating and cultivating *B. abortus*. Where contaminations were present he removed conditions favorable for their growth by using oxygen under tension in a sugar-free serum agar. He considered the method of Nowak as beset with more difficulties than sealing the tubes after the injection of oxygen, and described an apparatus for performing this. On suitable media the growth may at first be slow and poor with only a few organisms developing, but they may be readily educated to grow profusely. He demonstrated that material inoculated directly from the fetus to slanted serum agar gave no growth in two weeks' incubation, but if similar tubes were sealed with paraffine, growth might sometimes be observed after six days. The explanation offered was that the organisms by using the oxygen over and over again reduced it to the proper tension.

Bang considered the disease a uterine catarrh, the exudate being derived from the mucous membrane of the uterus. McFadyean and Stockman in their cases of abortion, produced experimentally and otherwise, described the cotyledons as soft, pulpy and of a gray, yellowish, anemic color. At times the exudate seemed to lie chiefly about

them, but in one instance, although the exudate was marked, the cotyledons appeared fairly normal. The membranes were extensively or only slightly involved. Once a square inch only, near the os, was diseased. Wall¹³ has demonstrated that the uterine tissue first involved in this disease varies according to the entrance of infection. When it is by the vagina the cotyledons become the primary seat of the disease; when by way of the blood the mucous membrane of the uterus is first involved.

Bang noted the appearance of immunity after the first or second abortion.

Agglutination did not take place in normal serum in a dilution of one to twenty-five (McFadyean and Stockman) nor one to twenty (Zwick). In the diseased animals agglutination was obtained in a dilution of one to five hundred, one hundred and twenty-four days before term according to McFadyean and Stockman, and even from one to one hundred to one to ten thousand, according to Zwick. Gronsted,¹⁴ working with Bang, was able to immunize rabbits until their serum had an agglutinative power of one to six hundred. He found that the serum of cows suffering from this disease had an agglutinative power from one to twelve hundred to one to six thousand seven hundred, occurring even two months before term. The technic of complement-deviation has also been developed and proves more delicate than the tests by agglutination.

McFadyean and Stockman, bethinking themselves of the mallein and tuberculin reactions, proceeded to make a similar filtrate which they called "abortin." They injected ten cubic centimeters subcutaneously and if at the eighth hour the animal's temperature was 104° F. or more, the test was considered positive. Zwick stated that his results in this line seemed encouraging, but to date were too limited to be of importance. Immunizing has been fairly successful. Hesse¹⁵ has been able to obtain some favorable results by injecting the bacterial extract.

THE INOCULATION DISEASE IN GUINEA-PIGS.

In December, 1909, we received material from a valuable herd of dairy cattle. Abortion had occurred in two or three instances and material was sent to the laboratory in the hope that means of prevention might be found. A bit of cotyledon of the placenta of Cow 218 (number given the animal in this laboratory) was shaken in normal salt solution and one-half cubic centimeter of this fluid was injected subcutaneously into a guinea-pig. The animal to all appearances remained in perfect condition and, having gained forty per cent in weight, was killed eleven weeks later to furnish sterile tissue for some anaërobic culture media. It was then found that the spleen was abnormally large as were also the lymph nodes. The case was therefore investigated with some detail and besides cultures other guinea-pigs were inoculated with its tissues. Although the cultures on various media remained sterile, the guinea-pigs in every instance developed marked enlargement of the spleen and lymph nodes. The virus was thus kept alive by passage through guinea-pigs. All attempts to grow the etiological factor outside of the body failed, but in one instance a bit of spleen transferred to a fermentation tube of ordinary bouillon caused clouding of the media when incubated. A fairly large anaërobic bacillus was demonstrated, evidently a contamination. In fragments of the inoculated tissue, groups of a minute bacterium were seen, but in later transfers this organism could not be found. After four transfers extending over a period of eleven weeks and again after four more transfers, making a total period of twenty-seven weeks from the original culture, one cubic centimeter of this fluid injected into a guinea-pig caused typical lesions.

It became of interest to know whether we were dealing with an ultramicroscopic virus and filtrates from shaken suspensions of infected organs were injected, but no disease developed.

At this time the work of MacNeal and Kerr¹¹ came to our notice and a culture of *B. abortus* was obtained from them

in order to determine if the etiological factor of this guinea-pig disease was related to it. Agglutination tests showed no clumping with normal guinea-pig serum, even in a low dilution of one to ten, but in the serum of a diseased pig the clumping was marked in dilutions up to one to one thousand. MacNeal's Culture I. (our laboratory number for this culture) was injected into guinea-pigs and caused the same characteristic lesions already noted. About this time pure cultures of a similar organism were obtained from both the spleen and the liver of two of the original series (Culture II. a and b). One of these animals had been inoculated with the contaminated culture, the other directly from the source of that culture. Further agglutination tests of these organisms thus isolated confirmed our previous results, for the clumping was complete in the serum of the animal inoculated with *B. abortus* I. in a one to one thousand and slightly in one to fifty thousand dilution. Thus we were dealing in all probability with *B. abortus*. We will now go somewhat more into detail concerning the material studied.

Cow No. 218. — This cow aborted at the eighth month and portions of the placenta were not received at the laboratory until six days afterwards. An odor of creolin or some other disinfectant was perceptible. Several of the cotyledons had a yellowish appearance but were still firm. Others were much softened. Smears showed the presence of three or more species of bacteria. The cells were very fatty. From this case Cultures II. a and II. b were obtained.

Cow No. 232. — The placenta in this case came from an abortion at seven months and was only forty-eight hours old. It was the second case which had occurred in a herd in a week. The veterinarian was uncertain whether to call this "Contagious Abortion" or not, as the cow gave birth to three calves (triplets), the first abortion having occurred at three and one-half months. The cotyledons were soft, yellowish, and easily scraped to pieces. Smears showed the presence of a fungus, numbers of minute coccus-like organisms, and some large bacilli. *B. coli* and a streptococcus were isolated by culture. To avoid confusion it might be said that *B. subtilis* was not employed in cultures from this case, and guinea-pigs inoculated with the tissue gave no characteristic lesions. We considered this abortion not due to *B. abortus*.

Cow No. 233. — Abortion occurred at the seventh month. The material consisted of a considerable part of the placenta and a small amount of turbid fluid. The odor suggested certain kinds of cystitis, but there was

no putrefaction. Smears from the cotyledon showed several groups of minute bacilli after much search. No other bacteria were seen. Culture III. came from this case.

Cow No. 235. — The placenta came from a six months' abortion. The tissue presented a very edematous, gelatinous appearance and contained about fifteen cotyledons; odor, inoffensive. The cotyledons varied in size and in the amount of injection and infiltration of the margins. The central portion of the cotyledon presented a yellow fringe of villi, but in some this had disappeared and there was a punched-out ulcer with a "shorn beard" base. Two or three cotyledons were markedly injected. Between these numerous superficial ulcers one centimeter in diameter appeared, slightly opaque and having a yellow granular appearance. Smears showed a few minute organisms and a bacillus present. From this case Culture V. was isolated.

Cow No. 234. — This specimen was the vaginal discharge following an abortion at eight months, and consisted of tenacious mucus of brownish hue containing debris such as straw and hair. Few organisms could be seen in smears but a staphylococcus was isolated. From this case Culture VI. was obtained.

In addition to these cultures there were studied a culture obtained from Professor W. J. MacNeal, of the University of Illinois, growing on a tube of blood agar which was designated Culture I., and a culture from Professor Bang of Copenhagen, Denmark, in capillary tubes of serum bouillon and also on serum agar. This was designated Culture IV.

The material to be inoculated was prepared in the following manner: In the case of a placenta one or two cotyledons were removed and washed thoroughly in normal salt solution, after which they were placed in sterile Petri dishes. With a sterile knife the yellow villi and basement membrane were scraped off and added to a small amount of salt solution. This suspension was shaken and was then ready for inoculation. The vaginal discharge was diluted by adding a small bit to salt solution, and was then well shaken. In the case of infected guinea-pigs, pieces of the spleen and liver and sometimes the kidney and lymph nodes were removed aseptically and ground with salt solution in an agate mortar or Latapie machine, and injected. Filtrates were obtained from the ground tissues by shaking the suspension for one hour and then filtering it through a Berkefeld filter.

With tissue suspensions one-half to five cubic centimeters were injected and gave positive results in practically every instance (four exceptions mentioned later). Of course the

actual number of *B. abortus* injected was very uncertain as the prevalence of the organism in the tissue was not known. If contamination with other bacteria was suspected, smaller amounts more diluted were injected in the hope that the animal's resistance would dispose of the few extraneous organisms inoculated.

With cultures the amounts could be better standardized. One cubic centimeter of a twenty-four to forty-eight-hour bouillon culture was usually injected. In one instance one-tenth cubic centimeter of a forty-eight-hour culture gave a typical pathological picture in ten weeks. Two cubic centimeters have also been injected. In a few cases a concentrated suspension of a culture was prepared for injection by scraping off the growth from a forty-eight-hour agar slant with a platinum wire and adding two and one-half to five cubic centimeters salt solution. Within certain limits the larger the dose the more marked the lesions. In our tissue suspensions a minute amount caused hardly any lesions, while five times that amount resulted in a marked disease. Naturally the injections with cultures were more uniform. However, after a period of eight to ten weeks the lesions were similar whether one or two cubic centimeters of a twenty-four-hour culture had been injected.

Most of our inoculations have been intra-abdominal (70 per cent), but the subcutaneous route has been used in many instances (25 per cent). Apparently the latter is slightly more severe and the initial loss of weight is greater. Intra-thoracic injections (4 per cent) were made to study lesions occurring especially in the lungs. The development and course of the disease is not influenced by the site of inoculation. Suspensions of cultures introduced by mouth have also caused lesions.

Signs and symptoms. — In a few instances a local lesion developed from a subcutaneous inoculation in which the amount of culture injected was very large and once or twice from a direct injection of cotyledon tissue. In these cases an induration appeared, sometimes extending over an

area two or more centimeters square. This later came to a head and discharged, or on section presented a yellow, granular, creamy mass consisting chiefly of mononuclear cells. If an ulcer did not form the mass usually became partially absorbed, but never much incapsulated. Ordinarily injections with pure cultures subcutaneously have not been followed by any local lesion except for a small swelling lasting a few days.

The animal may lose five to thirty and even one hundred grams in the first week—the larger the animal the greater the initial loss of weight. If the dose has not been too large this loss is slowly made good and the increase thereafter is normal or somewhat retarded. A considerable portion (72 per cent) of our animals showed some loss from their best weight when they were killed. This was generally a question of ten to fifty grams,—in one case four hundred and forty in an animal weighing seven hundred grams when inoculated. She died after thirty-six weeks.

A few days after inoculation the temperature rises usually about a degree, and continues uninterruptedly for twelve to twenty weeks or more, and then subsides. Much handling tends to increase the temperature one or two degrees. An animal which has a temperature of 103° or 104° F., by being handled may run a temperature of 105° to 106° F. Near death it becomes subnormal.

As a rule the animal presents no characteristic appearance. Three of our cases suffered from blindness. Swellings in the extremities especially about the carpal joints were noted. Weakness in the posterior limbs developed, associated with constipation and soiling of the parts. Recovery usually followed. These signs in one or two instances might be attributed in part to involvement of the joints or enlargement of the adjacent lymph nodes interfering with the movements of the limbs. In one case already referred to as losing so much weight, an inguinal lymph node became two centimeters in diameter and very firm, and the posterior limb was held abducted ten to twenty

degrees. After several weeks this swelling entirely disappeared. There was one case of paraplegia. The various lesions are apparently painless.

As regards the course of the disease the animals divide themselves into three groups. Some run a gradually downward course especially after the injection of a large dose, become much emaciated and die. The larger animals hold their weight relatively better. With an ordinary dose, such as one cubic centimeter of a twenty-four to forty-eight-hour bouillon culture, the animal usually gains, but often slowly. In other cases the normal physiological increase in weight continues. Some of our animals lived months and were then disposed of in good condition. At present we have one in apparently good health which was inoculated with virulent material sixteen months ago.

Undoubtedly certain guinea-pigs have more resistance to *B. abortus* than others. In our experiments four animals which were injected with suspensions of infected tissues had comparatively slight lesions at autopsy, and the organism could not be isolated from their tissues, although further inoculations from two of the four cases produced characteristic lesions.

Of the fifty-eight animals considered, twelve died. Death in two was due to rupture of the spleen; in four, to subsequent injection of toxins of the abortion bacillus, and in the remaining six it followed emaciation and exhaustion, kidney disease being extensive in two and pneumonia in one. It might be said, therefore, that only in five cases (8 per cent) did death occur as the uncomplicated result of inoculation with *B. abortus*.

Gross pathological changes. — These refer to animals, fifty-eight in number, inoculated with the original material, tissues of infected pigs, and cultures and which lived at least six to eight weeks.

The external appearance of the body may be normal or markedly emaciated. The opacity of the cornea of one or

both eyes and the swelling of the extremities have been already mentioned.

On reflecting the skin over the ventral surface there was usually fat tissue in slight amount. In large animals it was marked in some cases. In the emaciated every particle may have disappeared, exposing most beautifully the minute structures, such as nerves and vessels.

The lymph nodes were enlarged in fifty-six cases, or ninety-five per cent. The enlargement was chiefly universal, but occasionally some groups of nodes were more distinctly enlarged than others. The superficial lymph nodes, as a rule, were always larger than the rest; sometimes those in the inguinal region, sometimes those in the axillary or the cervical region were the most conspicuous. Of the internal nodes the retroperitoneal were always conspicuous and in some cases markedly enlarged. The nodes were perhaps more readily visible on account of the diminution in the fat tissue. They were frequently one centimeter in length, two or three millimeters in cross-section, and weighed .15-.20 gram. The color was a delicate, translucent pink. On section no definite structure could be made out. The cut surface was moist and succulent. In a few instances slightly opaque areas were suggested, focal in character, or extending along the periphery, and undefined extraneous blackish pigment was occasionally present. The mesenteric lymph nodes were enlarged and presented the characteristics already described. Those along the spinal column were of a much lighter color than the others.

Even before the abdominal cavity was opened in many instances the much enlarged spleen could be readily detected, occasionally extending well toward the pelvis. In a few cases it could not be seen until the organs were manipulated, being bound down by adhesions of a firm character. These were to the abdominal wall, liver, stomach, pancreas, and in two cases to the kidney. These adhesions occurred only in cases injected intraperitoneally.

The organ was enlarged in fifty-seven cases, or ninety-eight per cent. The one case in which the spleen was

normal in size lived thirty weeks before being chloroformed and showed other lesions of the disease. Enlargement was seen as early as the second week. In general, the spleen was of normal shape, averaging about 4.5 centimeters in length, 2.5 centimeters in width, and 4.6 millimeters in thickness. Instead of a normal weight of .4-.7 gram it averaged 2-4 grams, — once 9.9 grams. In many the distention of the capsule was conspicuous, the organ fairly bursting; in fact, in two instances, once while the cage was being cleaned out and once when the animal was being weighed, death resulted in a few minutes as a result of rupture. The surface of the organ may be normal or present numerous fine, grayish, pin-point elevations. Rarely there was an irregular cauliflower appearance as if from some internal new growth. This appearance was undoubtedly masked now and then by the great distention of the organ. Occasionally a few or numerous grayish, opaque, pin-head foci could be distinguished just beneath the capsule. The color was darker than normal. On section, the surface was very moist; the pulp usually swollen, soft, obliterating in part the normal markings. Seldom were distinct foci seen. When the disease became chronic the spleen appeared only moderately enlarged and rather flabby.

The liver was diseased in forty-three cases, or seventy-five per cent. In two or three instances there were adhesions to the surrounding tissues, again following intraperitoneal injection. The size of the organ did not seem to be definitely affected by the disease, and in chronic cases it may be small, although occasionally it had a tense, congested appearance similar to the spleen. Scattered over the surface were few or many grayish, translucent, pin-point foci just visible, or two or three millimeters in diameter extending into the substance of the organ. When newly formed these foci had a glistening, pearly appearance and later assumed a yellowish, opaque color. Rarely they had an opaque center and translucent periphery. The surface might also present a yellowish tracery or even distinct scarring. The gall-bladder

appeared normal. The hepatic lymph node was sometimes conspicuously enlarged.

The pancreas showed no marked changes. The islands of Langerhans were often quite conspicuous as yellow opaque dots of irregular size.

The kidneys were diseased in seventeen, or twenty-nine per cent of the cases. Of these, two had been inoculated with Culture I., twelve with Culture II., one with Culture V., and two with Culture VI. It is interesting that five of the twelve cases were injected with tissue from one guinea-pig. The inoculations were by both the intraabdominal and subcutaneous routes, and even with as small a dose as one-half cubic centimeter of a twenty-four-hour bouillon culture. Lesions were noted as early as the sixth week. The size of the organ varied only slightly from the normal as a rule. On stripping the capsule in a few instances definite round, grayish foci, similar to those in the liver, were noted. These protruded slightly, and were of a white color. In other cases they were much more indefinite and diffuse. On section there was rarely any radiating from these foci toward the medulla. Other round foci were seen in the substance of the cortex. In one instance both kidneys were distinctly enlarged, colorless, and apparently very diffusely and completely diseased.

No macroscopic lesions were found in the adrenals.

In only one instance was any definite lesion seen in the female genitals. In this case, at a point on the left horn about a centimeter above the bifurcation, there was a distinct nodule one centimeter in diameter bulging into the peritoneal cavity, with a surface closely resembling the cauliflower appearance seen on the spleen. The ovaries are not affected in our cases.

The testicles showed changes in ten, or forty per cent of the males injected. Five of these animals were inoculated subcutaneously and five intraabdominally and even one-half cubic centimeter of a twenty-four-hour bouillon culture caused lesions, the earliest found, occurring nine weeks after inoculation. All the strains caused the disease except No. V.

The testicles were adherent to the sac in three cases, all intra-abdominal inoculations, and associated with other abdominal adhesions. The testis proper was fibrous in two cases. The epididymis was involved in eight cases; the right in three, the left in three, and both in two cases. On removing the testicle from its sac, the epididymis when diseased was distinctly enlarged and presented one or more yellowish areas which on section allowed soft, yellow, granular material to escape. This opaque material was seen twice, in part of the seminal vesicles of the affected side.

In the lungs lesions were noted in thirty-eight, or sixty-five per cent of the cases. These did not involve the pleura but lay just beneath. They might take the form of pin-point, translucent, grayish dots more or less thickly sown, and resembling colonies of a pure culture, or a tracery of irregular shape, or distinct solid areas often contracted and depressed, involving a third of a lobe. On section the surface was fairly normal, disclosing firm, irregular, grayish areas only in a few cases. In one case the pleura was affected secondarily from disease of a rib.

The bones were involved in sixteen cases (27 per cent). Five of these had been inoculated subcutaneously and with only one-half cubic centimeter of a twenty-four-hour bouillon culture in two instances. Slight lesions developed in the ribs in one case after three weeks, in the extremities in another after five weeks.

The vertebræ were involved in two cases. The lesion in one of these occurred at the junction of the lumbar and sacral portion without causing any apparent deformity, and was discovered on investigating a yellow discoloration beneath the dura of the spinal canal. On section of the bone a small amount of yellow pus oozed forth. The other involvement was in the body of the sixth dorsal which bulged three to four millimeters into the thoracic cavity. Upon section a small amount of yellow, granular material was contained behind a shell of bone.

The disease of the ribs was most interesting. It was caused by one strain (No. II.) in seven of the eight cases,

and, as in the disease of the kidney, several were the result of inoculations from one infected guinea-pig. Strain No. 5 caused the eighth case. There were from one to five ribs attacked in any one case. These might be on the same side, adjacent or not, or on opposite sides. As a rule they lay between the fourth and seventh ribs. The disease was characterized by swelling which seemed to have its origin at the epiphysis adjoining the sternal cartilage. From this, extension and enlargement of the rib occurred, reaching in some cases to the vertebral column, but only once involving it. Different stages might be seen in the same animal (Plate XXI., Fig. 1). The swelling at the cartilaginous extremity, usually pink, was sometimes yellowish and occasionally it was on the point of rupturing into the thorax. Similar yellow swellings were occasionally seen along the course of the rib. The diameter of a diseased might be several times that of a normal rib (Plate XXI., Fig. 2). On section the medullary portion appeared larger than the whole of a normal rib. The tissue about the diseased rib seemed to be dense and fibrous; the rib itself less brittle than normally. The marrow instead of having a pink normal color was yellow and quite soft. Involvement of the pleura from a discharging rib was seen once as already mentioned; in another case the lung presented a depressed area at the diseased point.

In ten cases the extremities were involved; the carpal region in eight, the tarsal in one, and the knee in two cases. One animal after fourteen weeks had disease of the vertebræ, ribs, knee, and carpi. The lesions of the extremities appeared as rounded, fusiform swellings, and on removing the skin presented a smooth, pale, translucent, fibrous surface, except in a few instances in which yellow points protruded between the tendons (Plate XXI., Fig. 3). Section through the joint showed that the lesion was chiefly one of the distal portion of the bone and the cartilage, involving the joint only by extension. In one instance of disease of the knee joint the leg was rigidly extended. Section of this joint showed no destructive process but a dense, fibrous thickening about the joint.

Blindness was observed in three cases, one of the left, one of the right, and one of both eyes; all from Strain No. II. The cornea was grayish opaque. The earliest case occurred after thirteen weeks. A few cases of weakness in the posterior limbs associated with constipation and soiling of the parts developed, but usually this was only a temporary condition and the animal recovered. No nerve lesions were seen. The one case of paraplegia developed seven weeks after inoculation with original material and was chloroformed five days later. On exposing the lower cord, the lumbar enlargement seemed swollen. The dorsal median blood vessel was markedly dilated. The remaining portion of the cord and brain seemed normal. No cases have shown brain lesions.

No lesions of the muscles, heart and digestive tract have been seen.

Histological changes. — To avoid repetition it seems best to describe first the cellular elements common to the various lesions. It may be said that the essential changes associated with this disease are of a chronic, inflammatory character, and resemble those of tuberculosis, often to a startling degree, both in character and focal dissemination. The lesions are small, usually microscopic, and occur chiefly in the perivascular areas in most of the viscera of the body.

The focal lesion consists of a group of epithelioid elements, *i.e.*, cells with large vesicular, usually oval nuclei, poor in chromatin. The cytoplasm stains very poorly and is ill-defined. Among these cells may be a few nuclei with large peripheral blocks of chromatin and probably lymphoid cells.

The numerical relation between these two kinds of cells varies from case to case and from organ to organ. Some foci are composed mainly of large cell elements, while the periportal and perivenous cell masses are largely of lymphoid elements. In some foci, plasma and giant cells may be noted. Occasionally polynuclear leucocytes are seen. Mitoses are numerous. In the more chronic cases the tissue

changes have progressed beyond the stages described and signs of organization into connective tissue are present.

In the lymph nodes foci of epithelioid cells may make their appearance as early as the tenth day after inoculation. Later they become more profuse and resemble tuberculous tissue closely. These groups of cells seem to push aside the lymphoid elements which remain to the last at the periphery (Plate XXI., Figs. 4, 5). Occasionally a focus of these cells lies directly under the capsule, bulging it slightly. Extension into the surrounding tissue may occur (Plate XXII., Figs. 1, 2). An occasional giant cell and groups of plasma cells may be seen. The connective tissue hyperplasia becomes very pronounced in the lymph nodes, the fibers forming a network about the nests of epithelioid cells but later occupy the greater part of the node, especially the central portion. Different nodes in the same animal may present different stages of infiltration or organization. Rarely focal necrosis is present.

In the spleen one is attracted by the great dilatation of the blood sinuses which might be expected from the macroscopic appearance. Cellular proliferation is active as may be seen by the frequent mitoses. The epithelioid cells occur in groups as has been described and involve the organ more or less extensively. These may invade the Malpighian bodies or lie just beneath the capsule as in the lymph nodes. Polynuclear leucocytes are occasionally quite numerous. Giant cells are usually rare but may be present in large numbers, the nuclei arranged peripherally. Phagocytic cells may or may not be numerous. Rarely we have found cells containing typical bacilli when stained with aniline-water gentian violet. The connective tissue changes are not as marked as in the nodes.

In the liver characteristic cell foci may be seen scattered diffusely through the tissue, especially at the border of the lobule in close relation to the blood vessels. The subcapsular location is also common in this organ. The foci may be composed of a few cells or occupy an area the size of two or more lobules. The liver cells are pushed aside or become

included and degenerate, the protoplasm staining a diffuse, eosin red. Focal necroses are common in some cases. Polynuclear leucocytes are present, and rarely a few isolated giant cells. Dilatation and proliferation of the bile ducts is noted. The lymphoid cells are in increased numbers in the periportal regions. Connective tissue appears about the larger areas.

In the pancreas the islands of Langerhans may be greatly increased in size, but no focal lesions have been seen. In one or two instances, extension from a neighboring lymph node has caused atrophy and fibrous tissue changes.

In the kidney infiltration of the perivascular space between the cortex and medulla is quite striking, consisting usually of lymphoid elements (Plate XXII., Fig. 3). Scattered through the cortex, and occasionally subcapsular, are more or less definite focal areas composed of groups of epithelioid and lymphoid cells, chiefly the latter. These infiltrate the interstitial tissue, compressing and destroying the convoluted tubules and glomeruli (Plate XXII., Fig. 4). The process may be very general and diffuse. Proliferation of both types of cell has been seen in the papillary extremity of the medulla. Mitosis is present to a moderate degree. Subcortical depressions may be present, due to foci which contain connective tissue elements.

A few small foci have been noted in the cortex of the adrenal, just beneath the capsule.

Sections of the uterus from the case described macroscopically showed infiltration of the muscle with typical epithelioid cells. These force their way between the fibers and form large nests of cells, the central portion being prone to degeneration and necrosis (Plate XXII., Fig. 5). Another case of uterine involvement was detected in sections only. No foci were seen in the ovaries.

Extensive proliferation of epithelioid cells and infiltration with lymphocytes have been seen within the stroma of the testicle and epididymis (Plate XXIII., Fig. 1); sometimes one element predominating, sometimes the other. Connective tissue may replace the secreting tubules in great part.

Occasionally necrotic foci are seen composed of dense nuclear débris, the tubules containing masses of polynuclear leucocytes.

Infiltration of lymphoid cells about the blood vessels of the heart muscle has been seen (Plate XXIII., Fig. 2).

Scattered through the parenchyma of the lungs are irregular groups of epithelioid and lymphoid cells. These may lie just beneath the pleura or about a blood vessel or bronchus. The infiltration occurs in the interstitial tissue causing a small tubercle-like focus. It presents a central portion of epithelioid cells and a periphery of lymphoid cells (Plate XXIII., Fig. 3). Larger areas are composed of a mixture of the two elements. Mitosis is present and characteristic intracellular bacilli are to be found after careful search (Plate XXIII., Fig. 4).

In one area of a salivary gland a diffuse lymphoid cell infiltration occurred (Plate XXIV., Figs. 1, 2), and once a small, round tubercle was seen, in which the polynuclear leucocytes were conspicuous.

The thymus gland showed a small focus of epithelioid cells in one case.

From a study of our various sections of diseased bone it would seem that the primary lesion lies in the marrow of the epiphysis. The marrow becomes very vascular and the number of giant cells increased. At various points small, round foci of epithelioid cells develop (Plate XXIV., Fig. 3). As these foci coalesce and extend, the marrow fat spaces disappear, and the normal marrow is replaced by connective tissue. Development of blood vessels is marked. Mitoses are numerous and the marrow cavity becomes densely cellular, the number of leucocytes being large.

Already with the first changes in the marrow, signs of proliferation appear in the bone. The periosteum becomes very cellular and new bone is rapidly formed in a bold network, the Haversian canals containing large blood vessels (Plate XXV., Figs. 1, 2, 3). The osteoblasts are very numerous. Beyond the limits of active bone proliferation there is a diffuse and extensive connective tissue formation with

atrophy of the muscles. Internally the absorption of bone may be rapid.

The diseased vertebræ and ribs demonstrate the above process in its various stages. As the involvement of bone extends, the contents of the marrow cavity become more disintegrated and occasionally rupture occurs, and a yellowish, granular material is discharged, containing a large excess of leucocytes.

In the extremities proliferation of connective tissue occurs about the joints. The marrow of the epiphysis may be markedly diseased, and that in the diaphysis, separated by the epiphysial line, quite normal. The periosteum of the diaphysis may be very cellular and active, causing some new bone formation. The articular surfaces of the joint remain smooth.

The sclera, choroid, and lacrimal gland of the diseased eyes showed areas of infiltration, chiefly of lymphoid elements. Some typical tubercles were seen and also intracellular bacilli. Sections of cord, etc., examined to date have shown no lesions. In the one case of paraplegia the dorsal median vessel of the cord presented an infiltration of its wall (Plate XXVI., Fig. 1). An infiltration of a nerve ganglion adjacent to the adrenal was seen (Plate XXVI., Fig. 2).

No microscopic lesion of the skeletal muscles has been seen. Characteristic foci have been found, however, in the associated connective tissue and also in the adipose tissue.

Hypersensitiveness.—In order to learn whether the prolonged disease of the guinea-pig renders it hypersensitive to the toxins of the abortion bacillus, as is the case with the tuberculous guinea-pig, we have injected a suspension made in the following manner: A series of old agar slant cultures were scraped and the growth added to old bouillon cultures of various strains, forming an opaque, light brown fluid. This was allowed to freeze and thaw in the ice-box for about two weeks. It was then shaken for two hours on three successive days and finally it was heated on a water-bar

at 60° C. for half an hour and then centrifugalized, the clear fluid being used to inject.

Two cubic centimeters of this fluid injected subcutaneously caused death in a markedly diseased guinea-pig in seventeen hours, and even as small an amount as one-half cubic centimeter caused death in twenty-seven hours. Coryza and dyspnea were noted. If moderately diseased, the animal did not succumb until the fifth day after injection and in those mild or chronic cases in which the lesions seemed to be slight or quiescent only a local swelling occurred at the point of inoculation. This swelling which never ulcerated was composed of a soft, granular, yellow material, composed chiefly of mononuclear cells. In only one case was *B. abortus* cultivable from this material.

In the fatal cases the fever rose to 105° and 106° F. and became subnormal before death. The controls always had about a degree of fever for a week or ten days. No local lesion developed. The lesions seen post-mortem in an acute case were those of an intoxication: subcutaneous edema and hemorrhages; free fluid in the body cavities; congestion of the viscera, especially of spleen, liver, pancreas, and kidneys; hemorrhages into the stomach wall; liver and lymph nodes. In sections of the wall of the local lesion thus produced by the toxin there is a delicate layer of connective tissue externally, and internal to this, groups of characteristic epithelioid cells and marked proliferation of new blood vessels. Lymphoid cells are numerous and leucocytes in moderate numbers. Giant cells are rarely present. The central portion of such a lesion is composed of numerous, large phagocytic cells and débris.

SUMMARY. — *Bacillus abortus* may be said to cause lesions in guinea-pigs of a practically constant and most remarkable character. These usually appear between the third and sixth week (within ten days as determined in sections by the microscope), the acute changes extending over a period of ten to twenty weeks, after which reparative processes appear. The disease is accompanied by fever and tends toward final

recovery, though the animal may die from rupture of the spleen, emaciation, and exhaustion.

All the tissues of the body may be attacked with the exception of the muscles. This universality and frequency is best seen in sections under the microscope, as only the far advanced lesions are recognized by the unaided eye. In the tissues involved and in the histological changes produced, the disease closely resembles tuberculosis. The lesions have a predilection for the perivascular and subcapsular regions of the various organs. Injections cause at first a profound disturbance of the circulation in certain organs, notably the spleen, which becomes enormously engorged. Intra-abdominal inoculations are frequently followed by adhesions about the spleen. The localization of the disease in the testicle as well as in other organs, even when *B. abortus* is inoculated subcutaneously, is quite remarkable. The proliferation of bone although not common is extraordinary when present.

It was not our intention to test the ability of *B. abortus* to produce abortion when introduced into healthy pregnant animals. However, in one case the animal was pregnant and received one cubic centimeter of a forty-eight-hour bouillon culture (Strain No. III.) subcutaneously. Two weeks later she aborted. Cultures of the spleen of the embryo remained sterile. This animal died five weeks later as the result of a distended and ruptured spleen.

As this paper was being written our attention was called to an article by Schroeder¹⁸ on "Milk as a carrier of infection." He made the observation several years ago that a considerable percentage of guinea-pigs inoculated with milk to test the presence of the tubercle bacillus suffered from a curious chronic disease resembling tuberculosis in its gross pathological appearances. He was able to transfer this disease from animal to animal but was unable to isolate the organism. More recently he has observed a cow whose milk caused the same disease. With "specially modified culture media" he obtained a growth of a non-acid fast, very small, comparatively short bacillus. This organism is Gram-positive. Inoculations proved it to be the etiological factor.

When writing, he had under observation six cows giving milk containing this bacillus, although drawn so carefully as to exclude all external

contamination. Five per cent of milk samples tested contained this bacillus and ten per cent of a herd of one hundred and fifty animals showed the presence of the bacillus in their milk, although all the cattle appeared healthy. One cow at autopsy showed no udder disease and only slight liver lesions not yet studied.

The disease in guinea-pigs is characterized by a very large spleen; large, edematous lymph nodes with areas of degeneration; a swollen liver with numerous necrotic areas; and in males, by a more or less complete breaking down of the testicles. Two very unusual symptoms, paralysis and a peculiar joint disease, have occasionally occurred. The latter he believes due to a micrococcus which was isolated and which may be harmless when not associated with the bacillus. He thinks the disease has been overlooked, owing to the organism not growing readily on ordinary media, and the fact that macroscopic lesions may not appear in guinea-pigs until after six or more weeks. According to the present system of milk inspection this organism would not be recognized and would not be included in the present method of determining the number of bacteria. The thermal death point of this organism was 60° C. for fifteen minutes.

This disease corresponds so closely to the one that we have observed that it is most likely due to the same or a closely related organism.

Circular 198, Bureau of Animal Industry, issued March 2, 1912, by the U.S. Department of Agriculture, identifies the above bacillus as *B. abortus*, it having been demonstrated to be Gram-negative.

THE INOCULATION DISEASE IN OTHER ANIMALS.

Mice: White and gray mice have been inoculated intra-abdominally or at the root of the tail in thirty cases. The material inoculated has been one-half to one cubic centimeter of a twenty-four to forty-eight-hour bouillon culture; a turbid suspension of an agar slant, or the crushed spleen of an infected mouse. Many of the animals died from complications, frequently without apparent cause. Of the animals living nine weeks or more, there were nineteen, and of these thirteen, or sixty-eight per cent, showed distinct macroscopic lesions.

An enlarged spleen was common to all these cases and occurred also in a few of the other cases even after a period of three to four weeks. The organ was of normal shape but swollen, tense, and of darker color; weight from three-tenths to seven-tenths gram. The lymph nodes were enlarged in very few instances and presented no focal lesions. Of the other organs the lungs were apparently diseased in a few

cases. Irregular pneumonic areas, sometimes of a light grayish color, were noted. Occasional indefinite light areas were seen on the kidney surface, but not much importance was attached to these as infection with *coccidium klossiella muris* was common. In one of the mice having the disease six months a whitish area was present on the margin of a liver lobe and both epididymi were much enlarged.

Microscopically the spleen showed moderate distention of the sinuses, but not as marked as in the case of guinea-pigs. No epithelioid-like foci were seen. A diffuse hyperplasia was apparent from the many mitoses. In the lymph nodes this hyperplasia was also prominent. The liver presented many sub-endothelial roundish or elongated masses of lymphoid cells. Other small round foci occurred in the parenchyma. The whitish area on the liver margin proved to be an infarction of degenerated liver cells. The lesion cutting off these cells was of a chronic fibrous character containing numerous newly formed blood vessels and a rare giant cell. The character of the cells strongly suggested *B. abortus* as the cause. The foci in the lung were composed chiefly of lymphoid cells and occasionally groups of larger embryonic connective tissue cells. The kidneys, even when free from *Klossiella muris*, contained numerous foci of lymphoid and plasma cells scattered through the cortex, usually perivascular. The diseased epididymi were the seat of an extensive infiltration of lymphoid elements invading the interstitial tissue and rarely the tubules themselves. By the blocking of a duct a large cyst had been formed which was full of phagocytic cells, spermatozoa, and débris. In the adipose tissue adjacent was a focus of round cells. In another section a similar focus contained two giant cells (Plate XXVI., Fig. 3). From serial sections this focus was seen to be in close proximity to a blood vessel.

It is highly probably that *B. abortus* produces lesions in mice. With small doses (.5 cubic centimeter of a twenty-four-hour bouillon culture) they may occur after a period of a few weeks. Constant disease may be expected with somewhat larger doses. The method of inoculation is immaterial.

After a longer period (three to six months) diffuse lesions of a chronic inflammatory character ensue and become extensive.

Rabbits: Ten rabbits have been inoculated with *B. abortus*. The injections have varied in amount from one cubic centimeter of a twenty-four-hour bouillon culture to a turbid suspension of a forty-eight-hour growth on slanted agar. Intravenous inoculations were made in addition to the routes already employed. In some instances repeated inoculations were made. A subcutaneous injection causes a local lesion, sometimes measuring two to three centimeters in diameter or there may be only a moderate indurated area along the course of the needle. No ulceration results and the mass may become partially absorbed and slightly encapsulated. On section it contains a yellow, granular material resembling that found in like lesions in guinea-pigs. The animal appears in good health at all times and gains weight readily after an initial loss of fifty to two hundred grams. Fever is not present.

The animals autopsied have not given any evidence of disease although cultures of the organism may be readily obtained from the spleen after a period of ten weeks or more. The spleen may be slightly enlarged.

Microscopically the organs appear normal. Sections of the subcutaneous swellings resemble those in guinea-pigs resulting from toxin inoculation. Around a necrotic center of phagocytic cells and débris is a layer of large epithelioid cells and a few leucocytes. New blood vessels are present. The connective tissue is in slight amount. In an animal living six months which received three injections a month apart, a suspicious round cell focus was seen in the papilla of the kidney.

Cattle: A heifer was inoculated intravenously with five cubic centimeters of a forty-eight-hour bouillon culture but remained well.

Monkeys, rats, and pigeons have been injected without showing any signs or symptoms up to the present.

CULTIVATION OF *B. ABORTUS*. — In our work, as in former investigations, the chief problem has been to obtain successful cultures from tissues. Of the methods enumerated we have followed that of Nowak with slight modifications. A small bit of the washed cotyledon is removed with sterile forceps and transferred to a slant of ordinary beef-infusion-peptone agar. This is ground and broken up with a flat, platinum wire against the side of the tube, and the surface of the medium is smeared with it. A loop of the condensation water is then transferred from this tube to a second, and from this to a third tube. In the case of guinea-pigs a bit of the liver, spleen, or kidney is transferred to agar as described above. Inoculations by stabbing the organs with a platinum wire have given satisfactory results, but when the organisms are relatively few they may be missed by this method.

From experience we find that the spleen and lymph nodes contain the most organisms. Then come the bone marrow, liver, kidney, and lung, in order. A cube of lung tissue, three to four millimeters in size, may only give rise to two to four colonies. For this reason we have used the spleen regularly.

The tubes inoculated from the cotyledon are incubated at 37° C. in the ordinary way for twenty-four hours and then any colonies marked which have appeared. The tubes are then placed in an ordinary quart fruit jar having a screw metal top and rubber washer. A slant of ordinary agar is inoculated with *B. subtilis* and placed in this jar with the other tubes. The jar is sealed by screwing down the cap firmly and it is then placed in the incubator at 37° C. for two or more days. By this time small, characteristic colonies have appeared on the surface of the media in addition to those already noted. By this method we have been able to

isolate *B. abortus* in pure culture from original tissues which we have had at our disposal, and in practically all the cases of guinea-pigs suffering from this disease. We have succeeded in isolating the organism from the latter after a period of thirty-seven weeks, but in these chronic cases one to eight colonies developed instead of thirty or more as is common when cultures are made after a period of ten to twenty weeks. More recently we have found that by merely sealing the culture tube with sealing wax, and incubating it longer in some cases, we have secured satisfactory results.

The action of *B. subtilis* was studied in some detail in an endeavor to find out under what conditions *B. abortus* grows most readily. For this work we used a normal salt solution suspension of the original culture. This assured the presence of *B. abortus* and controls demonstrated that growth by ordinary incubation was practically negative. It was found that an excess of actively growing *B. subtilis* prohibits the growth of *B. abortus*. One square centimeter of fresh growth of *B. subtilis* to seventy-five cubic centimeters of space in the jar gives a profuse growth in forty-eight hours; 1 to 125 gives a moderate growth. Even one square centimeter to seven hundred cubic centimeters of space gives a distinct growth, the control remaining sterile. The age of the bacillus *subtilis* culture shows a diminishing influence up to the fourth or fifth day, after which it fails to stimulate growth. The failure evidently coincides with the period of sporulation. The manner in which *B. subtilis* and *B. abortus* tubes are corked is immaterial, provided the diffusion of gases is not interfered with. The bacillus *subtilis* tube may be open; the cotton plug may be loosely adjusted or dipped in paraffine as is customary with solid media in this laboratory, or a tin-foil covering may be also added, without checking the profuseness of the growth. As a routine the paraffine-dipped cotton plug was left in place.

The effect of using a sealed glass jar alone without the culture of *B. subtilis* was next investigated. Undoubtedly in some instances this is an advantage. The temperature in such a jar rises more slowly in the incubator naturally, and

therefore the tubes do not reach the incubator temperature until some time after those placed in with them but not in jars. The atmosphere of the jar remains at a more uniform degree of temperature and moisture unless the incubator is opened but rarely. It was possible for us to obtain original cultures in some instances by means of the sealed jar alone, the controls remaining sterile. This method cannot be depended upon and in several instances the tubes failed to develop.

We next tested the advisability of growing *B. subtilis* and *B. abortus* together in a test-tube sealed with sealing wax. Apparently there was not enough oxygen present for our strain of *B. subtilis* to grow appreciably under these conditions and neither organism developed. When, however, the seal was broken, *B. subtilis* promptly multiplied profusely.

The following simple procedure was also found effective. One tube of *B. subtilis* and the first transfer from a *B. abortus* culture were connected with a piece of webbed rubber tubing fifteen to twenty centimeters long and with an internal diameter of thirteen millimeters, by adjusting the rubber tube tightly over the ends of the plugged tubes, thus forming an inverted U. *B. abortus* grew as profusely as by the method of Nowak.

If the action of *B. subtilis* is merely one of reducing the oxygen tension in the jar, other organisms should act in a similar manner, although perhaps not in the same degree. Several kinds of bacteria were therefore tested, using *B. subtilis* as a control, among them *B. coli*, *B. megatherium*, a staphylococcus and an undescribed bacillus of guinea-pig pneumonia. It was found that *B. coli*, *B. megatherium*, and the staphylococcus gave results similar to *B. subtilis*. The pneumonia organism, although growing as profusely as the others, was able to stimulate *B. abortus* only in a slight degree, for the growth was just visible. Similar tubes incubated in a sealed jar alone remained sterile. When forty-eight hours old these various cultures exerted no further favorable influence. None of these organisms seemed to give

a more favorable growth than *B. subtilis*, and we have therefore continued our work with the latter.

It was furthermore evident from our tests that different strains of *B. abortus* required different degrees of stimulation. Culture II., a and b, developed on blood serum without any aid. Some strains could be grown in a sealed jar; others by slightly more stimulation, as with the pneumonia organism, and still others required *B. subtilis*. Whether still other strains require still more favorable environment remains to be tested.

As the action of the above organisms seems to be one of diminishing the amount of oxygen, we attempted to produce similar results by the use of alkaline pyrogallol. This was employed in glass jars and in Buchner tubes in varying amounts. With the former we attempted to produce partial anaërobic conditions. In the latter we varied the amount to include various degrees of anaërobiosis; *i.e.*, a series of tubes were inoculated in the same manner with *B. abortus* (first transfer) known to grow readily with *B. subtilis*; the dry cotton plugs were pushed in about two centimeters and dry pyrogallol in amounts ranging from .001 to .5 gram were added, and then one cubic centimeter of a ten per cent solution of sodium hydrate, and the tube quickly sealed with a rubber stopper. Prolonged incubation gave no growth save in the control tube, in which it was profuse in seventy-two hours. After four or five days one of the other tubes was uncorked and placed in a jar under the influence of *B. subtilis*, and *B. abortus* developed readily. These experiments show the need of very carefully adjusting the tension of oxygen.

In our experiments we used ordinary agar media, but we also tested agar made from extract of meat in place of the meat infusion, and also agar made plus glycerine. Apparently meat infusion agar is more favorable for growth but, with a stimulant like *B. subtilis*, growth on the other two is as profuse. We have made various experiments with ordinary agar by adding various quantities (drops) of dextrose or sodium carbonate or both, and also by growing *B. coli* on

the medium for twenty-four hours and reslanting it before inoculation. No growth has occurred as a result of these changes in the media.

Other conditions of growth were ascertained after the second or third transfer, when the organism grew moderately without the use of *B. subtilis* under ordinary conditions. In one or two instances we obtained growth from the first transfer however.

Fermentation tubes of ordinary bouillon inoculated with *B. abortus*, after an incubation of two to seven days, showed marked clouding of the bulb with a sharp line of demarcation at the junction of the branch. In other words, the organism gave pronounced evidence of being a strict aërobie from the first.

It was found that if ordinary bouillon tubes were inoculated and placed under the influence of *B. subtilis* the bacilli multiplied much less readily than on agar slants. This retardation could be overcome in some measure by using a broader surface of medium, as for example, a thin layer in an Erlenmeyer flask.

Plate cultures of *B. abortus* after several days' incubation showed minute colonies on the surface and just beneath. With prolonged incubation other colonies developed somewhat deeper in the medium. These latter were much smaller than those at or near the surface and remained so.

Shake agar cultures gave a profuse surface growth, but in no instance did it extend beyond two or three millimeters beneath the surface. A similar growth was obtained in the agar-gelatine-serum of Bang when a readily growing organism was inoculated. No zonal growth was noted, only the surface and immediate substratum becoming cloudy. When this medium was inoculated directly from an original source no growth occurred. When, however, the tube was placed with *B. subtilis* in a sealed chamber, burr-like, white colonies developed throughout the medium seven to ten days after those grown on slanted agar had appeared. There was no zonal character to the growth and the deeper (4 centimeters)

colonies were the smaller. Deep agar inoculations treated in a like manner remained sterile.

At the time we attempted to grow *B. subtilis* and *B. abortus* in a sealed tube we also placed *B. abortus* alone in a sealed culture tube and were somewhat surprised to find a profuse growth after a rather prolonged incubation period (8-17 days). This method has been used extensively since and in every instance has proved as satisfactory as with *B. subtilis*. In some instances the results have been exactly the same. In others, the colonies have not developed for several days after the growth had appeared with *B. subtilis*, and the colonies remained smaller, but it was of interest to note that occasionally many more colonies developed on the sealed tube than on the one associated with *B. subtilis* (Plate XXVII., Fig. 1). As might be conjectured, the size of the sealed culture tube, as well as the number of organisms transferred, bears a distinct relation to the result. Ordinarily we have used tubes thirteen and one-half centimeters long and eight millimeters in diameter. Sealing the tubes with sealing wax or paraffine gives equally good results. Tubes supplied with small rubber stoppers are, however, more easily manipulated and as effective.

It is probable that strains will be found to vary more or less in their relation to oxygen and to the kinds of culture media usually employed. This is shown by our own experience with the six strains thus far studied. For instance, Culture II., a and b, we were able to isolate without *B. subtilis* directly from guinea-pigs inoculated with the original material. In this instance, the tissues of two guinea-pigs were broken up as described, and spread over slants of ordinary agar containing one-half to one cubic centimeter of defibrinated guinea-pigs' blood. After a period of some four days, pure cultures were obtained (Plate XXVII., Fig. 2). These two sub-strains have been kept distinct and their cultural characteristics studied.

Culture I. was received from Professor MacNeal on a slant of blood agar. A goodly amount was transferred to an ordinary agar slant and, although the growth was very feeble

at the end of twenty-four hours, in four days it was quite abundant, and no further difficulty was experienced in cultivating it on ordinary agar in air.

Culture IV. from Professor Bang was contained in capillary tubes of serum bouillon and also on serum agar. The serum bouillon culture was transferred to ordinary agar slants and also to agar containing a small amount of guinea-pigs' defibrinated blood. These tubes were then exposed to the action of *B. subtilis*, the pneumonia organism, and in a sealed jar by themselves. Upon the plain agar slants no colonies developed, but upon the blood agar with the action of *B. subtilis* a profuse growth was obtained in forty-eight hours, and with the pneumonia organism in seventy-two hours; the tubes shut up in the jar and those in the chamber of the incubator remained sterile. (Professor Bang in a letter stated that the bacilli grew in the old manner (specific sub-stratum), but that he had also found the bacilli grew on the surface at times.)

It will thus be seen that of these two cultures sent to us and grown artificially for some time, one grew very readily on our media, while the other required not only the action of *B. subtilis* but also the presence of blood in the media. This is the one instance where we have found the presence of blood necessary. It is quite probable that the manner in which they had been cultivated before coming to us was responsible for the differences noted.

We have also made experiments to find the quickest and most certain way of educating this organism to grow aërobically. Apparently it makes no great difference whether the organism be passed through a series of rapid transfers under the influence of *B. subtilis* or whether the first colonies developing unaided furnish the source for subcultures. The action of *B. subtilis* is beautifully shown in the following manner. If we smear the surface of an agar slant from a culture which requires the aid of *B. subtilis* to develop, and expose it to the action of *B. subtilis* for forty-eight hours, a profuse film of growth covers the entire surface. If a similar tube is allowed to incubate unaided it often remains sterile,

or one to four or five colonies only develop from the extremely profuse inoculation. These do not grow readily and may not appear for four to six days, but once started they continue to grow even at room temperature.

In the first transfers growth is rare, but in the second and third a few colonies usually develop unaided. After five to seven transfers the organism has reached a stage which is nearly constant, and a loop of fresh culture develops readily on an agar slant in forty-eight hours.

It is an interesting fact that after the organism has adapted itself to ordinary aërobic conditions it does not lose this characteristic by further passage through guinea-pigs. Some of our guinea-pigs so inoculated have lived fifteen to twenty weeks, and yet upon making cultures from the spleen *B. abortus* developed even at room temperature (Plate XXVII., Figs. 3, 4).

Some notes on the morphology and cultural characters of *B. abortus*. — The following statements apply to all the strains studied, as no differences have been noted. The bacillus has been described as being .6 to 3 μ long and .5 μ broad. Bang himself stated that the longest organisms were the length of tubercle bacilli. Our measurements of the bacillus are .6 to .8 μ long and .5 to .6 μ broad. There is some variation in length which in some individuals may be equalled by the diameter. This type suggests a coccus. Some individuals may be 1 to 1.5 μ long, especially in bouillon cultures, but this is rare. The diameter is fairly constant.

In smears from cultures the bacilli lie separately or in twos, end to end, and rarely six in a chain. When in small clumps no definite arrangement is noted.

There is no motility, although Brownian motion may be very pronounced.

The bacillus is readily stained by all the ordinary dyes, perhaps most distinctly with dilute carbol-fuchsin. The stains used were carbol-fuchsin (concentrated and diluted); aqueous methylene blue; Löffler's methylene blue; and aniline water gentian violet. The organism stains uniformly

throughout, although irregularities (some portions staining deeply and some feebly) have been noted by Preisz and Holth. The ends are rounded but occasionally the corners are not as deeply stained, giving the bacillus an ovoid appearance. Carbol-fuchsin seems to accentuate the diameter; gentian violet, the length. The organism is Gram-negative and is readily decolorized by weak acids after having been steamed in carbol-fuchsin. No capsule has been demonstrated and no spores are formed. In old cultures degenerated and involution forms may be seen. Preisz speaks of branched forms.

Cultural characters: The following characteristics appear common to all of the six different strains studied. Of these four were from Massachusetts, one from Illinois, and one from Denmark.

On agar, the colonies have been described as transparent dewdrops with dentated edges. Preisz described them as having a bluish-white luster. Later they became yellowish-brown and McFadyean and Stockman laid considerable stress on the "rusty brown" color. Under the microscope MacNeal noticed a few coarse granules near the center of the colony.

We observed numerous small colonies on agar plates after forty-eight hours' incubation. The surface colonies appear as convex droplets of rounded, sharply circumscribed outline, about one to one and one-half millimeters in diameter, very glistening, *i.e.*, reflecting light markedly, and of a semi-opaque whitish color, suggesting droplets of mucus. They have an iridescent, mother-of-pearl sheen. Under the low power of the microscope the surface colonies have a very fine stippled appearance, brownish by transmitted light, darkest at the center. The deep colonies are much smaller than those on the surface and are of wedge or lemon shape. With the hand lens they are seen to have a lighter margin or are faintly banded with lighter zones.

The surface colonies develop slowly but persistently, until in plates containing only a few organisms it is not rare to have a colony five millimeters in diameter, of a whitish

opaque appearance. In place of the stippling, droplets as if of air or water are seen, very numerous and just external to the central portion of the colony. The deep colonies grow slightly and become darker brown. The plates, sown with many bacteria, develop in two weeks a fine ground-glass appearance due to the innumerable minute colonies; again those farthest from the surface are the smallest. Those which have burst the bottom film of agar spread out on the glass of the Petri dish in a thin, almost colorless growth five or more millimeters in diameter. They appear much lighter than the surface colonies macroscopically. If incubation is continued four weeks or more the principal change is one of density only. In the larger surface colonies the opaque points in the colony enlarge until readily seen with the hand lens, and extend into the glistening, mother-of-pearl peripheral zone.

On the agar slant after twenty-four hours a delicate, fine, granular film may be just visible on the inoculated surface. The margin is sharply defined and no extension occurs. After another twenty-four hours the film is much heavier, appearing as a translucent growth of characteristic luster. The border is well defined, steep, and slightly elevated, until the central growth increases, giving the appearance of a glistening, moist plaque. The water of condensation is moderately cloudy, the growth rarely extending out on the surface. An abundant, whitish sediment slowly forms. The growth increases in density for about a week, after which it remains stationary. When a few organisms only are distributed over the surface, the growth is slower, their presence not being recognized until after forty-eight to seventy-two hours. Development continues, however, for two to four weeks even at room temperature.

This slow growth, continuing until the colony is a relatively large one, seems characteristic. It has also been noted by us that when a few organisms are transferred to an agar slant, there is considerable variation in the size of the colonies, and after several days some of them may be twice the diameter of others. If these tubes be kept for several

weeks at room temperature, occasionally small daughter colonies appear on the glistening surface of an older colony, in some cases in such numbers as to give it a granular appearance. This is also seen on potato cultures.

When these strains were first isolated, and for some months afterwards, no changes in the medium were noted, but after having been cultivated artificially for a year it was seen that crystals develop in the agar which are probably due to the increasing alkalinity of the medium.

In an agar puncture the surface growth appears in forty-eight hours as a convex, opaque, whitish, glistening colony, three millimeters in diameter, sharply circumscribed. The growth along the stab extends one centimeter from the surface, and consists of fine, round colonies clustered together. After five days the surface growth may have extended to five millimeters in diameter but is not as convex. There is slight further extension along the needle-track, the deeper colonies being too small to be distinguished individually with the hand lens. After two or more weeks the surface growth dries out, presenting radiating lines and concentric depressions. Often the remaining surface of the medium is covered by a light secondary growth due to the moisture present which has inoculated the remaining surface. The average length of a stab growth remains at about one centimeter; in one instance (II. a) it extended to two and two-tenths centimeters.

If a tube of agar be inoculated while fluid and then cooled promptly, colonies appear on the surface within twenty-four hours. No zone of growth develops in the agar even after a period of two or three weeks. There is slight growth just beneath the surface, but in no instance has this been at a depth of more than three millimeters.

Cultures made on glycerine agar, and extract-of-meat agar, have not shown any appreciable variation from the above picture. Agar made from meat extract seems slightly less favorable than from meat infusion.

On blood agar prepared with defibrinated blood of horses, rabbits, or guinea-pigs there was no increase in the profuseness

of growth. After four to five days the inoculated surface presents a thick, grayish, greasy film, sharply bounded. The glass wall of the tube opposite the surface of the medium takes on a semi-opaque, smoky, greenish color which extends slowly to a height of three centimeters from the water of condensation, after several weeks of incubation.

On Loeffler's serum the bacillus grows readily but not as profusely as on agar. A delicate, circumscribed film with raised margin appears after twenty-four hours. The surface is finely granular and slowly becomes glistening with additional growth. In general, the appearances resemble those on agar.

McFadyean and Stockman obtained no growth on gelatine either at room temperature or at 37° C. Other investigators obtained a growth after four to six weeks.

Owing to the lowered temperature (22° C.) at which gelatine is incubated, growth on this medium is exceedingly slow, and on gelatine plates only after ten days may colonies be recognized with the microscope. They may not be readily seen with the naked eye until after three weeks, appearing as opaque, whitish droplets on the surface. With the hand lens they are very glistening and porcelain-like. Under the microscope other smaller colonies may be made out in the medium. The smallest appear as round, sharply circumscribed bodies with a delicate colorless stippling, the interior having a brownish tinge (with growth). The largest surface colonies have a brownish color, deepest at the center. As the colony develops, other small, blackish dots appear, forming almost a tracery in the brownish stippling. This blackish pigment is usually more concentrated at the center or may be uniformly distributed throughout. In some, distinct zones are seen, *i.e.*, a light margin of moderate width; a dark zone of similar width; a narrow light zone, and then a wide dark center. Besides the single colonies of distinct outline others occur, built up of fresh additions of growth or as the result of two organisms developing side by side. There are all variations from a round colony with a double rim at one point to slight outgrowths heaped up. After a

period of three weeks more, the plates sown with many organisms present a light, feathery cloud in which are numerous distinct colonies (surface), some, one millimeter in diameter. With a hand lens this feathery cloud may be resolved into colonies. The growth is chiefly at or near the surface, the deeper the colony the smaller it is.

In the gelatine stab, growth is slow and only after ten days to two weeks does a fine, white cloud appear along the puncture line with scattered colonies in the lower third. One or more small, opaque, convex, white colonies one to two millimeters in diameter appear on the surface. After seven weeks, growth still continues, although the medium is dried out. It may extend the whole length of the stab, and delicate, white, isolated colonies result. No liquefaction takes place.

On the agar-gelatine-serum of Bang, as was mentioned above, growth occurs only on the surface or immediately beneath it when a readily growing organism is inoculated. The fact that colonies are able to develop in the depths when the tube is incubated under the influence of *B. subtilis* and not in deep agar similarly treated is evidence that this medium is more favorable in certain respects.

According to Preisz, bouillon cultures scarcely became cloudy. McFadyean and Stockman had difficulty in obtaining a culture in ordinary bouillon direct from natural material but were more successful when a transfer was made from an agar culture. The addition of one per cent of grape sugar rendered the growth more luxuriant. Nowak was able to grow the organism in ordinary alkaline bouillon, the upper portion of the medium remaining clear until the tube was agitated.

In ordinary bouillon, prepared with fresh beef or veal and with an acidity of .8 to 1.2 per cent of a normal solution, after a series of forty-eight-hour transfers, a loop of a two to four-day old bouillon culture transferred to fresh bouillon causes very slight clouding after twenty-four hours' incubation, and on agitating a slight amount of fine white, powdery sediment arises. This clouding increases whether the tube

is agitated or not, and after a week the medium becomes turbid, remaining so for about a month and then gradually clears. The sediment covers the concavity to a moderate degree. It changes from a powdery to a flaky consistency and later becomes tenacious and stringy. As a rule no membrane or film develops, but if the tube be incubated for two to four days and then set aside at room temperature a white, delicate film may appear on the surface. In bouillon of an alkalinity of three-tenths to one per cent of a normal solution the growth is not as profuse.

If potato is inoculated from a bouillon culture, visible growth may not appear for two or four days. The outlines are irregular since the bouillon spreads over the uneven surface. A more uniform streak is made by inoculating from an agar culture. After twenty-four hours a delicate, white, glistening moist streak develops which slowly assumes a yellowish and after several days a brownish hue. The growth becomes elevated, glistening, viscid to the touch, with sharp, steep borders. The color in many instances is a chocolate brown and resembles, as McFadyean and Stockman have stated, the appearance of glanders on potato. Different strains may give slightly different shades of color, but by further subcultures on the same or other potato media variations from tube to tube are found in any one strain. The same daughter colonies noted in the isolated agar colonies are occasionally present on the potato medium. The inoculated potato itself may turn dark after about a week's incubation.

In milk, on incubating the organism with pyrogallol, Preisz observed coagulation with the separation of whey, but other observers, although obtaining good growth, have observed no coagulation, McFadyean and Stockman stating that when it was present a streptococcus was also found. Nowak obtained slight acid production in litmus milk. With two of his strains grown with *B. subtilis* the milk became distinctly red in eight days.

The organism grows readily in milk but not as profusely as in bouillon. After a week or ten days the normal acidity

is found to be lessened usually to three-tenths per cent of a normal solution, when phenolphthalein is used as an indicator. Litmus milk remains blue during a period of four to six weeks after inoculation whether kept in a large chamber of the incubator or under the influence of *B. subtilis*. There is no precipitation or visible alteration in the milk.

The presence of indol and nitrites cannot be demonstrated.

The optimum temperature is conceded to be 37° C. Preisz obtained no growth at room temperature although Nowak did. McFadyean and Stockman used a temperature between 30° and 37° C., an original isolation not growing at the room temperature, but they observed that once the culture had started it would continue to grow at room temperature. We have found the organism grows best at 37° C., and slightly even at 20° C.

Bang was able to isolate the bacillus from mummified fetuses and from material kept seven months in the cold. McFadyean and Stockman found material infectious at seven months, but not after the lapse of a year, nor could the organism withstand drying three days under calcium chloride. Preisz was able to rejuvenate a culture with pyrogallol after seventy days, and Nowak succeeded with agar slants after months; in deep agar after two years. According to Preisz the organism withstands exposure to 50° C. for thirty minutes but is killed at 55° C. in three minutes. McFadyean and Stockman exposed dry smears in an oven for one hour at 55° C. and later obtained a growth, but not after an exposure of two hours. In a water bath the organism withstood 55° C. for ten minutes but not 59° C.

When actively growing cultures on agar slants were left exposed to light in the laboratory for three months and then fresh slants made with large amounts only a few colonies developed. One strain died out entirely under these conditions. Bouillon cultures under the same conditions for a period of four months gave a growth.

Smears made by placing a loopful of a forty-eight-hour bouillon culture on cover-slips and keeping them under a

bell glass showed the presence of living organisms for seventeen or more weeks depending on the strain, but the older films require a week for development.

To determine the thermal death point, tubes sixteen millimeters in diameter were employed containing exactly ten cubic centimeters of bouillon of an acidity of 1.5 per cent. A forty-eight-hour culture of the organism was made and after the temperature of the bouillon in the tubes had been raised in a water bath to 45° C. for fifteen minutes, one of these tubes was inoculated with three loopfuls (loop 1.5 millimeter internal diameter) of a forty-eight-hour culture. The tube was slightly agitated and after a further exposure of ten minutes cooled in a vessel of ice-water and then incubated for a week at 37° C. Other tubes were inoculated in a similar manner at higher temperatures. It was found that different strains have slightly different thermal death points. From several tests it appears that Strain No. I. is killed by a temperature of 56° C., the other strains at 59° C. for ten minutes. Briefly it may be said that the thermal death point of the *B. abortus* is 59° C. for ten minutes.

Fermented bouillon containing one per cent of dextrose, saccharose, and lactose was used to determine fermentation capacities. No action was noted either in the production of acid or gas. Alkali production reduced the one per cent acidity of the medium to nearly neutral, and in some instances it became one-tenth of one per cent alkaline (phenolphthalein) in ten days.

As has been mentioned there is no acid production either in milk or sugar bouillon. On the contrary the organism regularly produces alkali. Further tests were made with ordinary bouillon and alkaline (.3 per cent) bouillon, and incubating the tubes in the large chamber alone or with *B. subtilis*. No acid production was demonstrated in the case of the alkaline bouillon, the reaction becoming more alkaline. This was relatively less than in ordinary bouillon as the growth was visibly less.

The cultural characteristics of this organism are quite

constant when once its ability to grow on artificial media has been established. Among these characteristics may be mentioned the glistening iridescent colonies on agar and the variation in their size; the colonies on and the non-liquefaction of gelatine; the slow growth in bouillon; the conspicuous pigmented growth on potato; and the organism's inability to ferment dextrose, saccharose or lactose, or to produce acid.

[In concluding, I wish to acknowledge my indebtedness to Dr. Theobald Smith for suggestions and assistance in this work.]

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EXPLANATION OF PLATES.

(When not otherwise stated, the material was obtained from guinea-pigs.)

PLATE XXI.

FIG. 1. — Disease of ribs. Note bulging at the epiphyses; points about to discharge.

FIG. 2. — Disease of ribs. Upper rib normal, lower diseased.

FIG. 3. — Left anterior extremity. Irregular swelling and yellow points.

FIG. 4. — x 45 diameters. Inguinal lymph node showing marked general infiltration of the tissue with epithelioid cells. Lymphoid elements at the periphery.

FIG. 5. — x 420 diameters. Preceding figure magnified. Nests of epithelioid cells.

PLATE XXII.

FIG. 1. — x 30 diameters. Epithelioid elements extending beyond a lymph node into the surrounding fat tissue.

FIG. 2. — x 375 diameters. Preceding figure magnified. Note epithelioid cells with faintly staining protoplasm and large round or oval nuclei, containing a very small amount of peripherally arranged chromatin.

FIG. 3. — x 50 diameters. Kidney. Round cell infiltration about blood vessel.

FIG. 4. — x 130 diameters. Localized lesion in the cortex of the kidney.

FIG. 5. — x 20 diameters. Uterus. Nests of epithelioid cells between muscle bundles. Central necrosis.

PLATE XXIII.

FIG. 1. — x 6 diameters. Testicle. Areas of cell proliferation. Connective tissue formation. Area of necrosis.

FIG. 2. — x 120 diameters. Infiltration near blood vessel in heart muscle.

FIG. 3. — x 375 diameters. Lung. Tubercle formation.

FIG. 4. — x 1,500 diameters. Lung. Bacilli in epithelioid cells.

PLATE XXIV.

FIG. 1. — x 130 diameters. Salivary gland. Round cell infiltration.

FIG. 2. — x 315 diameters. Salivary gland. Tubercle formation. Note leucocytes present.

FIG. 3. — x 50 diameters. Cross section of rib. Small foci of disease in marrow.

PLATE XXV.

FIG. 1. — x 6 diameters. Cross section of ribs. Extensive proliferation of bone in the diseased ribs.

FIG. 2. — x 60 diameters. Fig. 1. Small rib on right magnified. Note beginning proliferation.

FIG. 3. — x 60 diameters. Section of diseased rib under same magnification as Fig. 2. Note absorption of the original rib as well as the marked proliferation of new bone. Atrophy of the neighboring muscle fibers.

PLATE XXVI.

FIG. 1. — x 130 diameters. Round cell infiltration in wall of dorsal blood vessel, lumbar cord.

FIG. 2. — x 250 diameters. Small, round cell focus in a nerve ganglion.

FIG. 3. — x 375 diameters. White Mouse IV. Cervical region. Focus in the connective tissue.

PLATE XXVII.

FIG. 1. — Cultures of an original isolation from splenic tissue. Guinea-pig received an intraabdominal inoculation of one cubic centimeter of a suspension of cotyledons, Cow No. 235 (Culture V.). Chloroformed after sixteen weeks. Agar slant on left incubated under the influence of *B. subtilis*; on right incubated in a sealed tube. Appearance after five days. An agar slant inoculated in a similar manner and incubated unsealed, remained sterile.

FIG. 2. — Culture of the original isolation of Strain No. IIa. on blood agar, without the aid of *B. subtilis* or sealing.

FIG. 3. — Guinea-pig inoculated with Culture IIb. growing readily aërobically. Animal chloroformed at the end of ten weeks. Each agar slant inoculated with a bit of spleen. Tube on the left incubated with *B. subtilis*. Tube in the center sealed and incubated. Tube on the right incubated unsealed. After seventy-two hours note the characteristic growth on each slant. The tube on the right shows the ability of *B. abortus* to retain its artificially acquired power to grow aërobically even after remaining in the animal body ten weeks.

FIG. 4. — Culture obtained aërobically from a bit of spleen of an infected mouse. Animal inoculated with a readily growing culture (IV.) and chloroformed after two months.



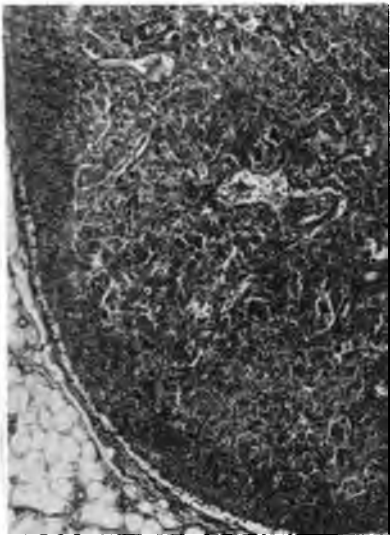
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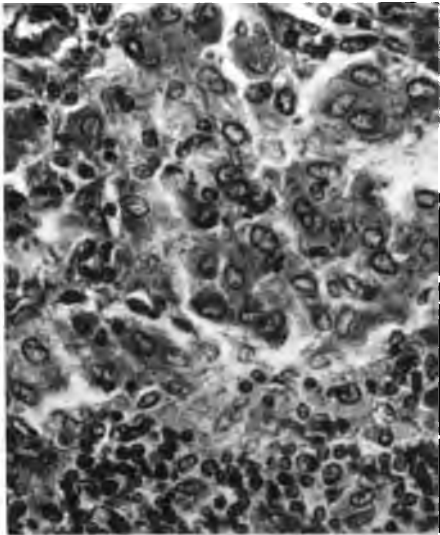
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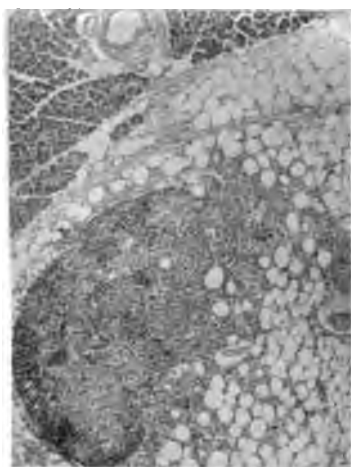
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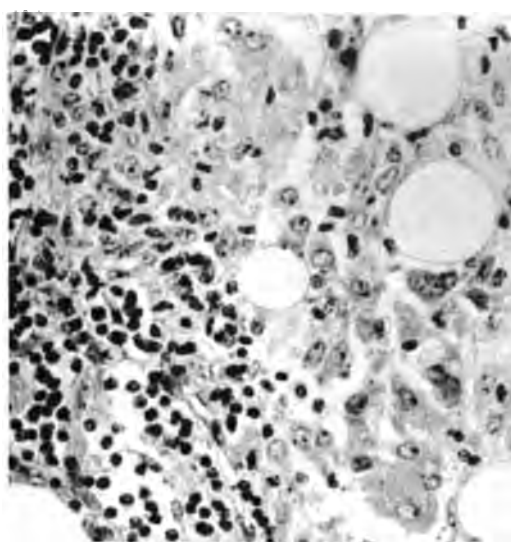
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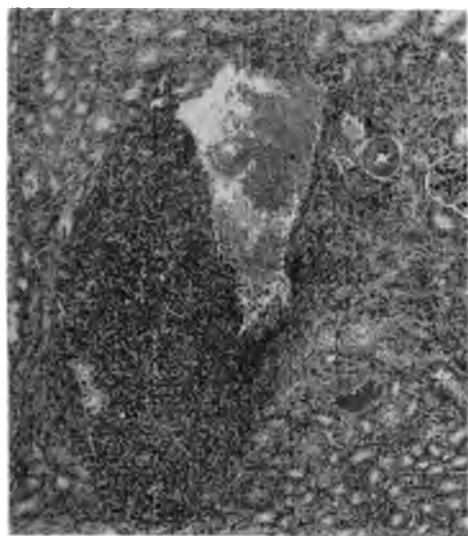
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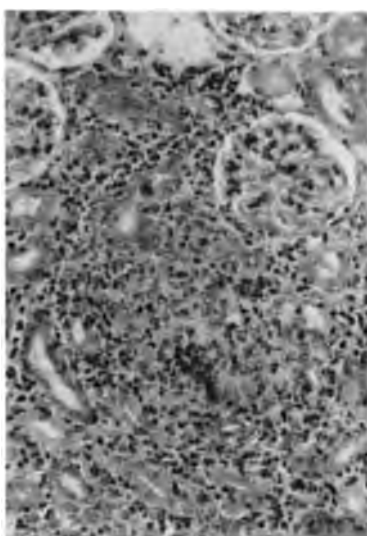
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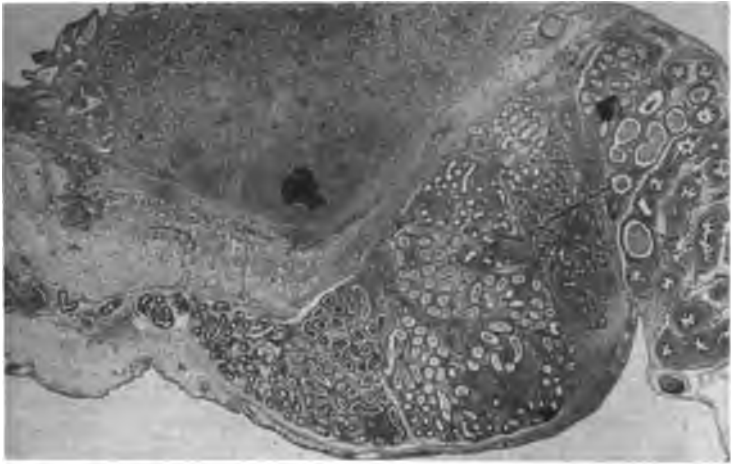


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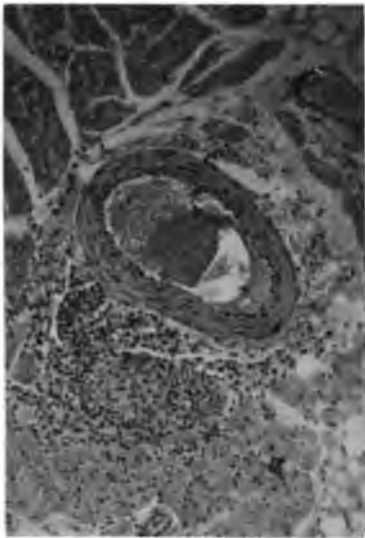


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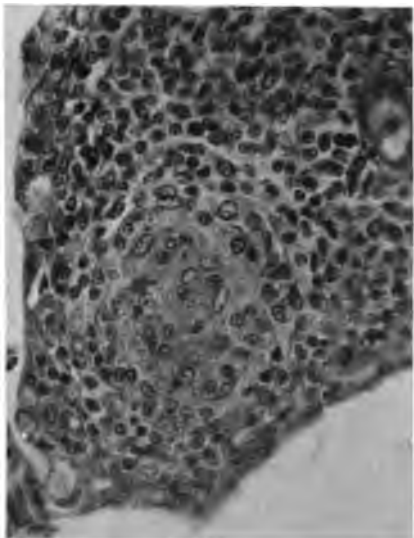




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Fabryan

B. Abortus

BACTERIUM OZÆNÆ (ABEL); FERMENTATION REACTIONS
WITH ELEVEN SUGARS, DIFFERENTIAL DIAGNOSIS, AND
USE AS A VACCINE FOR TREATMENT.*

CALVIN GATES PAGE, M.D.

(*Instructor in Bacteriology.*)

(*From the Bacteriological Laboratory, Harvard Medical School.*)

A swab received at the end of October, 1911, from a case of atrophic rhinitis, yielded a pure culture of a non-motile, capsulated, Gram-negative, rod-shaped organism. On agar it showed a moist, sticky, colorless growth. It made but slight acid in litmus milk. It produced gas in dextrose, saccharose, and adonite, but not in dulcite or inulin. It did not liquefy gelatine. The growth in bouillon was turbid without ring or pellicle. It produced no indol, and gave a negative Voges-Proskauer reaction.

From these data I was able to identify it as *Bacterium ozænæ* (Abel), as described briefly by Chester,¹ and to locate it as number forty-nine on the list published by Bergey and Deehan² in 1908. Abel's original description appeared in 1896.³

Since November I have been able to test this organism and some others with which I happen to be working simultaneously on eleven different kinds of carbohydrate.

Repeated tests have shown the following result, using Durham tubes each containing sugar-free bouillon plus one per cent of a single sugar. The mannite fermented first, the tube showing nearly five per cent of gas over night. At the end of twenty-four hours there was slight gas formation with dextrose, maltose, and raffinose. At the end of two days the gas had greatly increased in these tubes, while gas had begun to show in the tubes of dextrine and adonite. At the end of the third day the gas in the adonite tube increased moderately and in the dextrine tube slightly, but not until

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the end of the fourth or fifth day did gas appear in the tubes of saccharose or sorbose. The next day both of these increased very much, but not until another day or two was there any gas in lactose. Sometimes the lactose failed to ferment until the eighth or ninth day. No gas or acid ever formed in the tubes containing dulcitol or inulin. The percentage of gas varied somewhat with different trials, but the delay in the beginning of gas production with certain sugars was constant. The acid production was fairly parallel with the gas production.

I inoculated two guinea-pigs of equal weight with an equal amount of an emulsion of *Bacterium ozænæ*, one subcutaneously and the other intraperitoneally. The first had no apparent lesion or illness, and the other was found dead in the morning. Post-mortem showed extensive peritonitis. Cultures from the fluid and the organs showed abundant growth of *Bacterium ozænæ*. The heart blood smear showed numerous capsulated organisms. This harmless subcutaneous and fatal intraperitoneal guinea-pig inoculation agrees with Abel's experiments.

Abel found this organism in a hundred cases of atrophic rhinitis. He never found it in any other diseased condition of the nose, nor in any atrophic case after it had been thoroughly healed. In one case, inoculation of a sound nose with a culture started up the atrophic process. He did not always find it at the first trial, but after removing crusts it was usually possible to find the organisms in the fresh mucus. To get pure cultures it is absolutely necessary that the vestibule of the nose be sterilized, and the swab protected from contact with any surface or hairs.

The organism which most nearly resembles *Bacterium ozænæ* is the well-known Friedlander bacillus or *Bacterium pneumoniae*. This also has a capsule and makes but little acid in litmus milk for the first day or two.

Abel describes the difference between *Bacterium ozænæ* and *Bacterium pneumoniae* somewhat as follows:

The mass of growth in culture tubes is more watery or moist than the growth of the pneumonia bacillus. It is so moist that it frequently slides

down the surface of agar or serum to the bottom of the tube. In a gelatine puncture of *Bacterium ozænæ* the surface growth spreads out, while with the Friedlander bacillus the top of the growth heaps up, giving the so-called nail culture. The *ozena* bacillus never causes a brownish coloring of the medium in old gelatine or agar cultures as the Friedlander bacillus frequently does. The *ozena* bacillus gives no gas on potato, and makes but very little gas in ordinary gelatine or agar. The Friedlander bacillus is more apt to show short, coccus-like forms. Subcutaneous inoculation of a mouse with the *ozena* bacillus is always fatal, while the subcutaneous inoculation with the Friedlander is never fatal. Intraperitoneal inoculations of guinea-pigs would be fatal only in about half the animals used.

In 1906 von Eisler and Porges⁴ described a method of differentiating the Friedlander bacillus from *Bacterium ozænæ* and the Gram-positive rhinoscleroma bacillus by means of the precipitin reaction with immune sera. They show that these organisms have no common group reaction and hence are not closely related. Their method involves complicated laboratory technic and seems to me less definite than the differentiation by means of fermentation reactions.

Many different organisms have doubtless been included under the name of Friedlander bacillus and I feel sure that *Bacterium ozænæ* has sometimes been mistaken for it. Through the kindness of Professor C.-E. A. Winslow I received from the American Museum of Natural History in New York two cultures marked *B. pneumoniae*, one of which coagulated milk in two days and made no gas in dextrine or adonite. Evidently this is not the Friedlander bacillus. The other culture gives the typical growth and characteristics of the Friedlander bacillus, but it does not ferment dulcitol, which according to Dr. Bergey² it should do. It reduces neutral red more rapidly than does Abel's bacillus, and also reduces nitrate broth. I have tried it in parallel cultures with *Bacterium ozænæ*, and the results show in the chart and diagrams. While each ferments the same sugars, the delayed fermentation in *Bacterium ozænæ* cultures is very striking.

DESCRIPTION OF FIGURES.

FIG. 1. — Five of the eleven pairs of tubes drawn to scale to show amount of gas production. Each pair shows a decided difference. Mannite is perhaps the most striking and useful because differentiation can be made over night. Two-fifths actual size.

FIG. 2. — *B. osana*. Complete record of same experiment as shown in five left-hand tubes (Fig. 1). Mannite shows first and most active fermentation in this particular experiment. Fermentation of saccharose and sorbose delayed to fourth or fifth day; lactose to ninth or tenth day, finally reaching seventy per cent.

FIG. 3. — *B. pneumonia*. Complete record of same experiment as shown in five right-hand tubes (Fig. 1). Reactions mostly prompt, mannite especially so in this particular experiment. Lactose slow.

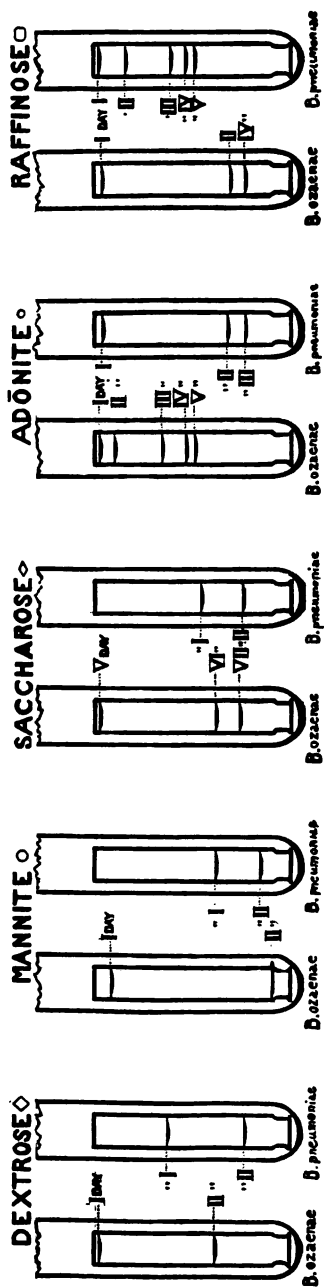


FIG. 1.

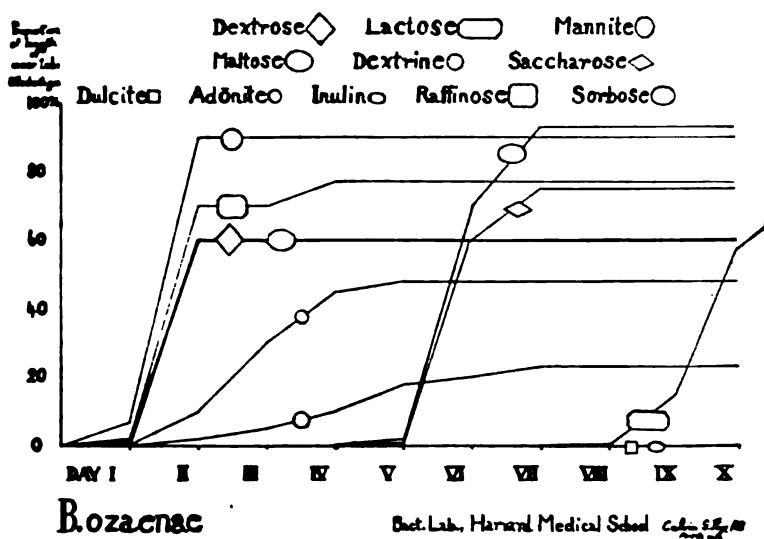


FIG. 2.

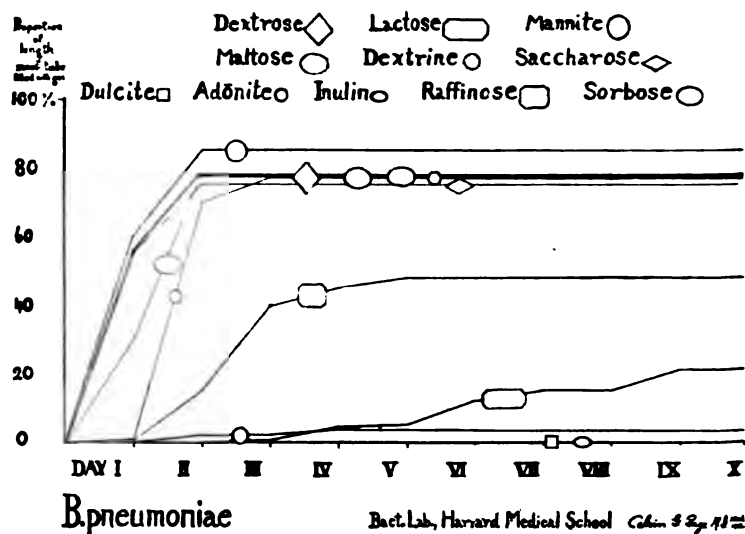


FIG. 3.

Now as to the method of preparing sugar media in Durham tubes. In order to get all the air out of the inner tube it is necessary to autoclave the tubes containing sugar-free bouillon, allowing the air to escape with the steam. I add the sugar after the tubes have been sterilized, because changes begin to occur in lactose and some other sugars when the temperature rises above 110° C. or six pounds pressure. Each sugar is made up in ten per cent solution in sterile water, sterilized twenty minutes in the Arnold sterilizer, and then added with a sterile pipette, about one-half a cubic centimeter for each tube. After steaming again for a few minutes, the tubes are incubated over night before being labelled with distinctive labels. For dulcitol and inulin I make the solution five per cent instead of ten because they are less soluble.

As dulcitol, sorbose, and adonite are expensive I have devised a small-sized tube which requires only a single drop of a ten per cent solution of sugar and one and one-half cubic centimeters of medium. When neutral red or litmus is added as an indicator there is no necessity for testing with litmus paper or making titrations.

The rhinologist sending the swab had had personal reports from London that a vaccine made from the Friedlander bacillus was giving good results in cases of atrophic rhinitis. I think that the two organisms possibly had been confused.

At the request of the patient and of the rhinologist I made a vaccine from the culture of *Bacterium ozænæ* and have been using it for treatment about every three days with gradually increasing doses. The patient noted continual subjective improvement, diminution of crusts and increasing moistness of secretion. After three months great improvement in the nose is reported and the belief is that the result is one of cause and effect and not mere coincidence. There has been no other treatment. A swab taken at this time showed abundance of Gram-negative bacilli with capsules, and culture yielded a pure growth of *Bacterium ozænæ*. The seat of the trouble in this case is in the right frontal sinus and on general principles one cannot expect to produce

a rapid effect through the circulation upon capsulated organisms growing on a mucous membrane with such limited blood supply. But some effect has been produced.

On February fifteenth I received from the same physician a swab from another patient — a man of forty who has had atrophic rhinitis with fetor for twenty-five years, but is otherwise well and vigorous. Culture showed *Bacterium ozænæ*, *Bacillus pyocyaneus*, a streptococcus and *Albococcus candidus*. I began treatment with vaccine previously made from *Bacterium ozænæ*. Since the fourth treatment I have injected also some *pyocyaneus* vaccine in the opposite arm. The local reaction corresponded in severity as the dose increased. After two weeks the patient noticed that his nose and throat were not so dry and the discharge, which was more moist, was diminishing in amount. On March twenty-sixth a fresh swab showed many capsulated Gram-negative bacilli in the mucus, and culture showed *Bacterium ozænæ* with very few colonies of *Bacillus pyocyaneus*. There has been decided diminution of the odor, and the patient is to continue with weekly treatments.

Cultures of *Bacterium ozænæ* recently isolated from these two patients show greater delay (9 and 15 days) in the fermentation of saccharose, but after passage through a rabbit one culture begins to ferment saccharose in three days. The characteristic delay with sorbose and lactose still persists. Two strains of Friedlander bacillus just received from another laboratory show minor differences from each other and the one mentioned above. One of them fails to ferment saccharose for twelve days. Acid develops with the gas, but in certain tubes the reaction later becomes alkaline.

In a recent article Borschim⁵ tells of extracting from the nasal crusts a proteolytic enzyme derived from the leucocytes and not from the bacteria. He also finds that *Bacterium ozænæ* has a soluble toxine and is making further investigations on this point.

Independent unpublished investigations made during the past two years confirm the value of vaccine treatment in cases of atrophic rhinitis.⁶

CONCLUSION.

1. The organism easily isolated from two patients corresponds with the description of Abel's bacillus.

2. In fermentation tests with eleven sugars, this organism shows delayed gas production with saccharose and sorbose, five days; lactose, eight days. No gas with dulcitol or inulin.

3. Several cultures from various laboratories marked B. pneumoniae ferment the same sugars, but without delay.

4. Autogenous vaccines made from this organism are giving encouraging results.

5. For makers of stock vaccines, fermentation tests offer a means of distinguishing Abel's bacillus from the bacillus of Friedlander.

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A CASE OF OVARIAN PREGNANCY.*

G. S. GRAHAM, M.D.

(*Assistant Professor of Pathology, Dartmouth Medical College.*)

The findings here reported have to do with a specimen which first came under the writer's personal observation some days after its delivery at the laboratory. When received shortly after operation the specimen had been sectioned and examined macroscopically, after which a part of the tissue had been thrown away and the remainder fixed in Zenker's fluid. It seems that while the discarded tissue was of comparatively small amount it undoubtedly contained valuable material, not least interesting of which appears to have been the embryo itself, since this could nowhere be found in the material available for study. Of larger moment is the fact that the question as to how embedding of the ovum took place cannot be determined excepting by inference, whereas a study of the accurately reconstructed gross specimen might, perhaps, have yielded more positive results. Despite such limitations, however, it is believed that as an undoubted case of ovarian pregnancy the lesion presents certain points of interest, particularly upon the side of histology.

The tumor mass was removed from S. E. R., married, thirty-six years of age. The patient is described in the hospital record as a fairly well developed and nourished woman who has never been strong though seldom ill. She has had two children and both pregnancies have been free from complications. The last child was born four years ago. She has never suffered miscarriage. The menses have always been regular, though for the past two years rather painful and profuse. She has just recovered from a month's constant flowing. This is the first instance of such intermenstrual flow that she has ever experienced. A little over one month ago there was an attack of right-sided abdominal pain associated with vomiting, the attack lasting for about four days.

Physical examination was negative excepting that there was tenderness over the lower abdomen, more marked to the left of the median line just above the pubes, while vaginally a small tumor was palpable in the region of the left ovary.

* Received for publication May 3, 1912.

On the day following admission to the hospital the appendix and the left tube and ovary were removed by operation. Recovery was uneventful.

The laboratory protocol gives the following gross description of the left tube and ovary:

The specimen consists of an ovary with its Fallopian tube. The tube measures about five centimeters in length and about .5 centimeter in its greatest diameter. Beyond an injection of the superficial blood vessels no evidence of pathological change can be determined. The fimbriated end is patent and from it depends a small appendix vesiculosa. The ovary is about the size of a hen's egg, reddish in color and rather soft. Its surface is irregularly furrowed or lobulated and appears free of adhesions. Section reveals a cavity about 1.5 centimeters in diameter placed excentrically within the ovarian mass and bounded by a thin white membranous wall of rather firm consistence. The tissue about this cavity is soft, reddish in color, and strongly suggests placental tissue. The surrounding ovarian tissue is firm and shows numerous injected blood vessels. In its thicker portion is a corpus luteum which borders upon the soft reddish mass above referred to and appears to be intimately blended with it.

Blocks of the preserved tissue were embedded in paraffin and in celloidin and sections have been stained by hematoxylin and eosin, eosin and methylene blue, and by Mallory's analin blue and phosphotungstic acid hematoxylin methods. Serial sections were made of a portion of the tissue.

Microscopic description.—Sections from the ampulla of the oviduct show a thin walled tube with a lumen 1.5 millimeters in diameter lined with a distinctly ciliated epithelium. A few scattered polymorphonuclear and large mononucleated cells lie in the recesses of the mucosal folds, but otherwise the lumen is free of cellular or other exudate. The tunica propria and muscularis show no evidence of past or present inflammatory process. The blood vessels of the outer muscular and of the serous tunics are dilated and crowded with red blood corpuscles. Grouped on one side of the oviduct near the outer margin of the external muscular coat there are found three longitudinally disposed tubules lined with columnar epithelium supported upon a delicate membrana propria, beneath which are bundles of smooth muscle cells arranged for the most part longitudinally but with occasional oblique strands. The lumina of the tubules vary in different sections from ten to twenty microns in diameter, and are lined by epithelial cells six to seven microns in

height and of columnar form. Distinct terminal bars are present and an occasional cell bears indistinct cilia.

The surface of the ovary is irregularly contoured and over a portion of it the germinal epithelium is well preserved. Within the cortex are found frequent corpora albicantia with an occasional Graafian follicle in an early stage of its development. The blood vessels are dilated and filled with erythrocytes. Many vessels of large diameter show only the simplest endothelial wall. There is slight endarteritis of the smaller arteries. From one block of the ovarian tissue, and from this only, there were obtained sections showing along the outer margin of the cortex a cross section of a tubular structure lined with a single layer of epithelial cells; these are usually of a high columnar form, although many cuboidal cells are mingled with the more typical elements. Scattered cells or groups of cells show distinct cilia and an occasional cell is found in mitosis. The main tubule has a diameter of from forty to fifty microns and from it are given off short lateral branches of smaller size. The epithelium rests upon a thin layer of fibrillar tissue beneath which there is a layer of varying thickness made up of typical stroma cells of the ovarian cortex and containing numerous capillary vessels, all deeply injected. A third layer of fibrillar connective tissue in which are embedded many smooth muscle bundles merges internally into typical ovarian tissue without the slightest appearance of any line of demarcation. This whole formation is first apparent in the region of the mes-ovarial stem of the ovary whence it extends outward for a distance of about one centimeter.

In general, sections through the ovary show an outer zone of typical ovarian tissue along whose inner margin a variable "transition zone" forms the boundary of a third and innermost zone. Within the latter are found numerous chorionic villi with intervillous spaces containing red blood corpuscles, fibrin, and trophoblast elements, together with a considerable number of leucocytes. The inner margin of this zone is defined by the fetal membranes. The outer zone of ovarian tissue is 1.5 centimeters in thickness at its

widest part; thence it tapers gradually to become in a part of its extent a mere capsule-like covering for the gestation sac. The thickest portion of the crescentic mass thus presented in sections encloses a recent corpus luteum with a relatively large non-obiterated central cavity. In its thinnest portion the ovarian ring consists of partially necrotic connective tissue with dilated thin-walled vessels and frequent interstitial blood extravasations, but as the body of the crescent is approached the stroma tissue becomes better preserved and normal histological details are defined. The layer immediately about the gestation sac is uniformly looser in texture than that of the outer portion of the ovarian wall and here and there appear in the interstitial spaces, particularly in the neighborhood of the blood vessels, large epithelioid cells with deeply stained, rounded or slightly irregular nuclei and dense cytoplasm, the latter at times slightly vacuolated. In the region immediately about the corpus luteum there is reached, finally, what may be considered as the attempted formation of a placental area. Here a rudimentary transition zone has become demarcated and along its inner edge is a varying layer of canalized fibrin while traces of a second deeper fibrin deposit are to be found within the stroma. The maternal tissue is invaded by cell columns containing occasional mitotic figures and derived from a proliferation of the cytotrophoblast covering the tips or sides of the villi. Such columns usually attack the stroma, where it overlies a superficial blood vessel, whence the invading cells advance beneath the vascular endothelium, dislodge it and push it inward, after which they spread laterally from the point of puncture as a single or double cell layer; the endothelium soon disappears and the maternal blood makes its escape through the breach thus established to enter the intervillous space along the sides of the cell column. Vessels may be punctured in this manner in several different places by cell columns from the same or different villi and occasionally the villus itself may enter the vascular lumen although this appears to be unusual. In case the invading cells find no blood vessel close enough for

successful attack their advance as a body ceases, but isolated elements may wander away into the surrounding stroma tissue where they congregate, particularly in or about the vessel walls; here their presence is often associated with the appearance of hyaline changes in the vascular connective tissue. In some cases the cells occur also within the vessels where they may appear as loose masses which may or may not be associated with the presence of similar cells within the vascular coats. A larger vessel of arterial structure lying close to the gestation sac appears to have repelled the attack of a cell column which had invested its walls but which failed to penetrate the muscular coat. The cells thus widely disseminated are of an average diameter of about twenty microns and possess a vesicular nucleus with deeply stained chromatin network and a prominent nucleolus; the cytoplasm is dense, finely granular, often with a slight basic tinge and with a delicate unstained peripheral zone of wavy or angular outline. Within the interstitial spaces the cells maintain these characteristics or show at most only a slight vacuolation of their cytoplasm, but among those found in the intravascular collections are many which are undergoing degenerative change, as is shown by an increasing vacuolation of their cytoplasm with consequent distention of the cell and by a progressive chromatolysis of the nucleus proceeding to a complete disappearance of its staining reaction.

Neither in the embedding area nor elsewhere is there any appearance of a stroma reaction comparable to that which results in the formation of the decidual cells of uterine placentation. The syncytium at times gains entrance into the stroma but never penetrates deeply. It is represented by an occasional multinucleated mass of cytoplasm which may appear to fade off into delicate rays of hyaline material, but the deep penetration of syncytial giant cells such as occurs in the uterus is never found. The syncytium may accompany the cytotrophoblast into the lumina of the maternal blood vessels and in one instance in which the connective tissue core of the villus had itself advanced into such a vessel the syncytium found lodgment upon the

internal vascular wall where it spread for a considerable distance along the denuded surface. Here it became vacuolated and a few of its intracytoplasmic spaces were filled with red corpuscles, a condition analogous to that to be described below as occurring in the intervillous spaces. For the most part the syncytium appears to lag behind when the trophoblast meets and invades the maternal stroma and while occasional masses or perhaps individualized cell forms may accompany the Langhan's cells, by far the greater bulk of the tissue spreads laterally over the surface of the stroma where it quickly undergoes degenerative changes and may be seen to fade off into a hyaline, non-nucleated material which is finally distinguishable from the so-called canalized fibrin forming the floor of the intervillous space.

The corpus luteum, about which the area of embedding seems to mass itself, has been made the subject of vigorous attack upon the side adjoining the gestation sac, where an area representing about two-fifths of the total area presented in sections has been destroyed, blood clot and an occasional chorionic villus, usually of poor preservation, entering the gap thus formed to mingle with necrotic lutein cells, leucocytes, and nuclear detritus. In some places the destruction of lutein cells has involved the entire parenchyma on the exposed side and the degenerative process has been arrested only after the laying bare of the central ring of loosely arranged fibroblastic tissue; again, an inner border of normal lutein cells may persist in an otherwise necrotic interseptal compartment. In the latter case there is often a sharply defined line of demarcation between the normal and the degenerated lutein cells. Degenerative changes in the lutein cells appear to be due rather to pressure from interstitial hemorrhage than to the solvent action of the trophoblast cells, since the latter are present only in small numbers within the degenerating areas, their cell groups never advancing beyond the marginal region of the original lutein cell formation while only rarely are isolated cells found within the deeper portions. The hemorrhage proceeds from vessels

of the theca folliculi or of the septa which have been punctured by trophoblast cells in the marginal region and as it forces its way among the lutein cells it breaks them up into islands or columns which soon undergo cloudy swelling. In eosin stained preparations the degenerating lutein cells present in many cases a uniform light brown pigmentation of the cytoplasm, while with phosphotungstic acid hematoxylin the cytoplasm is stained a deeper blue than is that of the normal cell. Subsequently there is shrinkage of the cell with granular disintegration of the cytoplasm and nuclear achromatosis. Early in the course of these changes the coarse fibroglia fibrils so abundantly developed in the normal cell become thickened and retracted so that they fail to pass beyond the boundaries of their proper cell but form a heavy marginal zone of deeply stained material about its cytoplasm. In the last stages of necrosis there is complete disappearance of the fibrils.

The fetal membranes present a typical amnion of flattened to cuboidal ectodermic cells variably intact or vacuolated and resting upon a narrow band of refractile non-nucleated mesodermic tissue. The terminal bars described by Freund and Thomé¹ cannot be determined, although a denser ectoplasmic border sometimes distinguishable gives an appearance at times suggesting such structures. Along the outer margin of the supporting mesoderm extends an interrupted layer of flattened mesothelial cells which frequently show cytoplasmic vacuolation and degenerative nuclear changes. The cavity of the extra-fetal coelom is not entirely obliterated but is bridged by delicate mesodermic strands. The chorion and its well developed villi are covered by a typical trophoblast within whose basal layer there is occasional mitosis, while at frequent intervals the syncytium sends delicate prolongations downwards between the Langhan's cells to the basement membrane. The syncytium does not contain mitotic figures; its cytoplasm is dense, finely granular in many places, and contains numerous small vacuoles which may appear colorless or of a faint blood-pigment coloration. The degree of vacuolation is variable and in general the

areas showing the greatest vacuolation are also those in which the "prickle edge" appearance of the outer syncytial margin becomes most prominent. Syncytial buds or proliferation nodes are often bounded on all their free surfaces by a denser border zone enclosing the central vacuolated cytoplasm within which the nuclei are embedded, and the prickle edge is here developed upon, or rather within, this marginal zone along its whole extent. The mesodermic supporting tissue of the chorionic villi varies in character from that of a well developed fibrillar tissue to that of a loose meshed tissue of embryonic type within whose spaces occur large vacuolated or granulated cells, the so-called Hofbauer cells. An occasional nucleated red cell may be found lying within the ill-defined blood vessels of the larger villi as has been noted previously in a few cases,^{2,3} although the finding contrasts with such statements as that of Bondi⁴ that neither fetal red cells nor fetal vessels could be discovered. In occasional instances degenerative changes of marked extent have involved entire villi.

The intervillous space contains red blood corpuscles, leucocytes, and other structures normally to be expected, as cell islands, proliferation nodes, and fibrin. About many of the villi there appears a space or channel of varying width outlined externally by a thin sheet of "fibrin" and filled with red cells which appear to be in various stages of disintegration. This appearance is particularly marked in sections stained by phosphotungstic acid hematoxylin. There is distortion of the size and shape of the erythrocytes and the individual cells show a tendency toward fusion into a common mass of homogeneous material. The suggestion of the progress here of some solvent action exerted against the red cell is supported by the fact that during the course of the morphological change the cells lose their strong affinity for the stain and stand out finally in sharp contrast with the normal cells circulating in the intervillous space. The envelope forming the outer wall of these perivillous blood spaces is at times connected with the syncytial layer of the trophoblast by interlacing non-nucleated strands of cytoplasmic

material undoubtedly derived from the syncytium itself. The whole appearance recalls the mesh-like syncytial figures described in the trophoblast of the early ovum such as that of Peters, while it accords also with a similar picture recently observed in this laboratory in the case of a trophoblast of about four millimeters diameter contained in a scrap of uterine curettements.

Discussion.— Among the previous reports upon ovarian pregnancy perhaps the greatest diversity of opinion has related to the identity of the large epithelioid cells found in the ovarian stroma about the gestation sac. They were described by van Tussenbroek in her classic paper and appear to have been interpreted by her as mainly of maternal origin. Subsequent observers have considered them as decidual cells, as decidually altered stroma cells, as endothelial cells, while again it is stated that they are indistinguishable from lutein cells. From the descriptions and plates of these various authors it would appear, however, that in all these cases the cells concerned have been, at least for the most part, Langhan's cells. Kühne⁶ considered as such the epithelioid cells observed by him in his study of tubal pregnancy and more recent writers have agreed with him in his conception of the Langhan's cell as an active invader of the maternal tissues. In the present case there is little difficulty in following the Langhan's cells from their position at the tips or along the sides of the chorionic villi into the ovarian stroma tissue, where they appear to exert some solvent action upon the maternal cells and particularly upon those of the vascular intima; thus they are enabled to penetrate the vessel walls, provided such wall is not too highly constituted (as is perhaps the case in the larger vessels), while as isolated elements they may wander through the stroma tissue singly or in small groups to attack the smaller vessels of the whole embedding area. While their action upon the more remote vessels appears to be limited for the most part to the production of a peculiar hyaline change in the vascular wall, it is possible that the intravascular cell collections above described

may come from a penetration of the intima by isolated groups of cells carrying behind them no solid chorionic villus to maintain the temporary breach made by them in the vessel structure. The close association of the cells with the vessel walls has no doubt led to their confusion with endothelial cells, while the failure on the part of many observers to trace the history of the Langhan's cell appears to be due to the fact that they did not find in their sections any actual contact of the chorionic villus with the ovarian stroma. Franz⁶ observed conditions entirely duplicated in the present case and reached conclusions as here stated in regard to the identity and function of the Langhan's cell.

Of larger moment is the question as to how the ovum secures implantation in the ovary. In the majority of cases it has been concluded that embedding takes place originally in the wall of a Graafian follicle. Van Tussenbroek and others have concluded that the impregnated ovum subsequently burrowed through the developing wall of the corpus luteum to take up its position in the stroma just outside. Hewetson and Lloyd,⁷ however, describe a perfectly intact corpus luteum protruding into the gestation sac and suggest, as does Franz for his case, that the ovum may have pushed its way downward into the stroma tissue from an original situation upon the surface of the ovary. On the other hand, Webster⁸ insisted that the ovum can implant itself only upon Müllerian tissue and believed that in all cases of ovarian pregnancy embedding takes place in rests of this tissue such as he believed easily demonstrable in the ovary during uterine pregnancy. Schickele's suggestion that implantation takes place in the epoophoron is in practical accord with this theory, as is also Leopold's contention that the ovum embeds itself outside the uterus only upon a mucous surface bearing cylindrical epithelium. In the present case the wide breach in the side of the corpus luteum reproduces the picture which has been interpreted as evidence of an outward migration of the ovum through the wall of that body, but such evidence in support of an intrafollicular embedding must yield before the finding of an epithelial lined tubule in

the cortex of the ovary. Even without a demonstrated communication of its lumen with the ovarian surface, the presence of this structure renders it in the highest degree probable that the ovum first found lodgment upon the epithelial surface here presented. On the other hand, given this original site of implantation, there still remains to be explained the fact that the gestation sac seems to be centered rather in the vicinity of the corpus luteum than about the epithelium of the cortical inclusion. Here it seems necessary that we postulate the existence of some attracting force exerted upon the embedded ovum by the corpus luteum, as a result of which the ovum burrows its way through the intervening stroma tissue to take up its position near the attracting body. The existence of such an attracting force would serve to explain the frequent close relationship observed between the gestation sac and the corpus luteum, while upon general theoretical grounds it would appear more probable that an ovum originally embedded elsewhere in the ovary might be attracted toward the corpus luteum than that after a primary embedding within that body it would attack the wall to pass outward into the more indifferent stroma tissue. From the appearance in the present case there can be little doubt that the attack upon the corpus luteum has been from without, since the area of degeneration appears as a cone with its base outward while its inner truncated apex still presents some residue of unaltered lutein cells.

The changes observed in the fibroglia fibrils of the lutein cells during the progress of cell dissolution are of interest. The morphological change in the fibrils occurs very early in the course of cellular change, so that it would appear that the condition of the fibril here becomes an index as to the viability of the cell to which it is appended.

As to the meaning of the epithelial structure found in the cortex of the ovary, there was a strong temptation to regard it as an embryonic rest of Müllerian tissue, particularly in view of the presence in the musculature of the Fallopian tube of structures probably to be interpreted as embryonic

remains; but the highly developed epithelium present, together with the size of the tube which it lines, and the accompanying wide layer of smooth muscle tissue point rather toward its being a small portion of the fimbriated end of the tube which has been caught to the ovarian surface by adhesions, twisted off from its original connection, and incorporated into the ovary. It may be noted in passing that previous writers have in some cases found more smooth muscle in the ovaries examined by them than would normally be expected to occur in that organ, and it may perhaps be questioned whether in these cases there may not have been presented some undiscovered structure similar to that here described.

Cases of ovarian pregnancy would appear to offer excellent opportunity for the study of the fetal elements, since the ovum is usually in an early stage of development and the picture is not complicated by the presence of uterine tissues. But with the possible exception of their testimony in support of the fetal origin of the syncytium they have contributed little of value. The embryo itself has seldom been found, probably, as here, through inadvertence, and when found it has been of no particular interest. Mesodermic and ectodermic tissues of the membranes and chorionic villi as well as the contents of the intervillous space are of normal appearance. The syncytial network above mentioned as surrounding the chorionic villus in such a way as to form a channel filled with disintegrating red cells is, however, of some interest. This net is not considered as a mere "symplasma syncytiale" indicative of degenerative change in the trophoblastic epithelium. It occurs perhaps most frequently toward the ends of the villi, where the trophoblast is most active, and here it appears to receive into its channels the blood corpuscles escaping from nearby vessels of the embedding area. The erythrocytes found within the spaces of the structure show morphological and tinctorial alterations which may be interpreted as indicating gradual solution of the cytoplasm, while the syncytium forming the wall of the space shows evidence of considerable

glandular activity by the presence within its cytoplasm of vacuoles and mitochondria. From such considerations the structure is interpreted as a net designed for the imprisonment of the red cell while it is being subjected to some lytic action preparing it for use as pabulum by the embryo. Such direct utilization of the red cell as an "embryotrope" during the early nutritive phase of the ovum is well proved upon other grounds despite the slight histological evidence that has been produced in its support. In the present case it may be conceived that the ovum is deprived of the usual nutritive material normally supplied by the endometrium and has been obliged to make greater efforts toward utilization of the red cell and its products than is necessary in the case of the early uterine embryo. The same consideration may account for the fact that here, as in previous cases, the syncytium shows in many places a highly developed "prickle edge," a structure probably to be considered as a brush border indicative of glandular activity rather than as due to the presence of cilia upon the syncytial surface. The simultaneous presence of a "hemotrophic phase" of nutrition in the present specimen is, of course, not to be excluded, since many villi are bathed directly by the histologically unaltered blood making up the bulk of the contents of the intervillous space. The trophoblast of such villi is typical of that found in the early uterine placenta. If the above view is correct, however, there may be in ovarian pregnancy an unusual persistence of the embryotrophic phase of nutrition and a histological demonstration of the utilization of the maternal red cell as an embryotrope.

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DESCRIPTION OF PLATES.

PLATE XXVIII.

FIG. 1. — Langhan's cells pushing into vessel and dislodging endothelium.

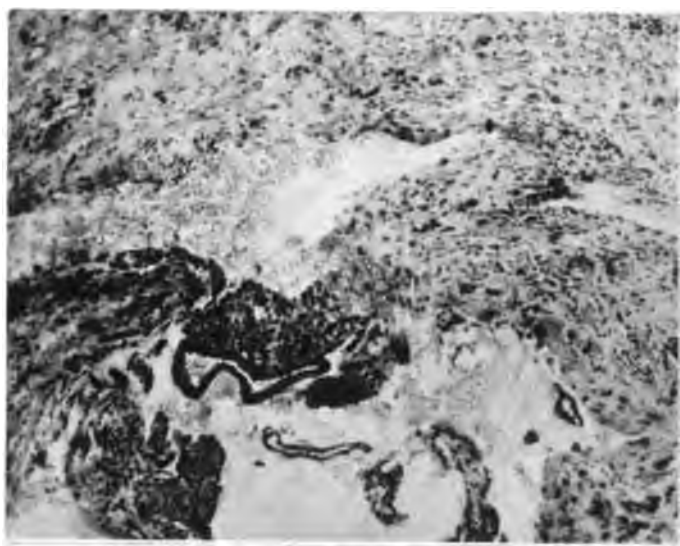
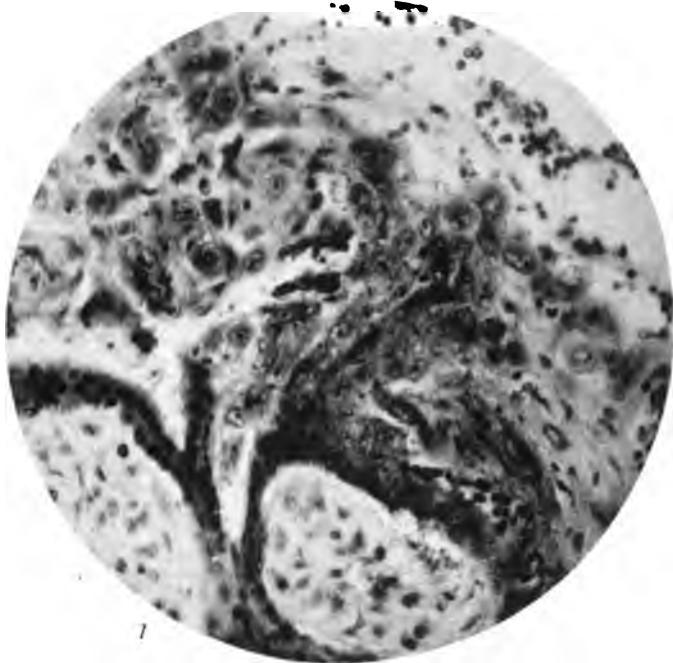
FIG. 2. — Trophoblast puncturing a vessel and spreading along its denuded intima.

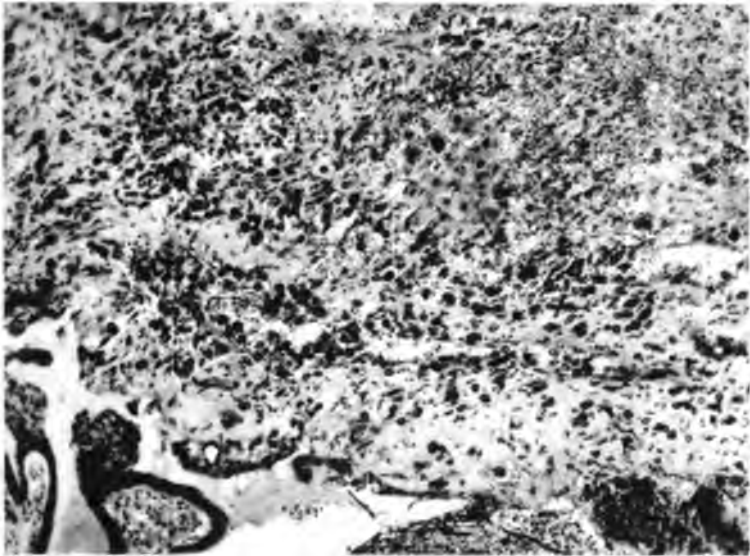
PLATE XXIX.

FIG. 3. — Edge of corpus luteum. Advance of Langhan's cells followed by hemorrhage. Latter leads to degeneration of lutein cells (mechanical).

FIG. 4. — Normal lutein cells showing fibroglia fibrils.

FIG. 5. — Degenerating lutein cells.





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ON INDUCED VARIATIONS IN BACTERIAL FUNCTIONS. AN
EXPERIMENTAL STUDY.*

A. C. ABBOTT, M.D., Sc.D., D.P.H.

(*Professor of Hygiene and Bacteriology, University of Pennsylvania.*)

(*From the Laboratory of Hygiene, University of Pennsylvania.*)

In 1891 I recorded an observation to the effect that if *Staphylococcus pyogenes aureus* be exposed to corrosive sublimate solutions of the strength of 1 : 1,000 for a time not sufficiently prolonged to kill all the exposed organisms, many of the colonies developing from the surviving individuals were of unusual brilliancy in color and revealed a peculiar viscosity that differentiated them markedly from the creamy colonies characteristic of the normal organism.¹

No explanation for this phenomenon was offered at that time nor is it possible to supply a satisfactory one now. It can only be said that among the survivors from such exposures there are always a few of the viscid colonies. If, however, such colonies be again exposed to sublimate solution they regain their normal creamy consistency after about the fifth successive exposure. This phenomenon is not peculiar to cocci exposed to corrosive sublimate solutions but occurs after exposure to several other metallic salts as well, notably copper sulphate.

In more recent efforts to obtain light on this matter certain other changes in the general behavior of the organisms have been noted from time to time — thus: as the exposures were continued the surviving colonies seemed gradually to acquire an intense orange color and, in the case of a few transplantations to gelatine, to be more active in their liquefying function than had been the case at the start. This led to a more or less systematic study of the influence of corrosive sublimate and other agents upon the commonly observed functions of *Staphylococcus aureus* as noted in the course of routine laboratory study.

The results, though of no apparent practical value, we

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regard as of sufficient general biological interest to justify a record of them.

In casting about for materials with which to begin work we selected for our first series of experiments (begun in October, 1910) a strain of *Staphylococcus aureus* that might fairly be regarded as normal in all respects, except that its power to liquefy gelatine was somewhat lower than that commonly seen with this organism.

For the second series (begun October, 1911) we selected, because of experiences gained in our first series, another strain that was conspicuously feeble in so far as proteolytic activity was concerned. Both strains came from suppurative processes in man, and both caused typical abscesses in the kidneys and myocardium of rabbits after intravenous injections.

As the experimental procedures were the same throughout, a general description will suffice.

Methods.—From a well developed agar agar culture of *Staphylococcus aureus* enough was carefully broken up in about one cubic centimeter of sterile sodium chloride solution (.85 per cent) to make a dense milky suspension.

About one cubic centimeter of this was then added to fifty cubic centimeters of the solution of the chemicals to be used. In our studies we employed mercuric chloride, phenol, sodium carbonate, and a number of the organic acids, though the results here recorded refer only to the mercury salt, the phenol, and the sodium carbonate.

As a rule the mercuric chloride was employed in 1 : 1,000 strength; occasionally it was weaker than this but never stronger. Phenol was for a time used in one per cent strength but it was finally reduced to .75 per cent as more favorable. Sodium carbonate was always a watery solution saturated at room temperature.

All the experiments were made at the temperature of the room.

The time of exposure of the organisms to the chemicals varied with the different agents: for sublimate it ranged

between five and twenty minutes, occasionally being as long as twenty-five minutes; for phenol solutions the exposures were for about the same time; for the sodium carbonate solution it was as a rule longer, rarely below fifteen or twenty minutes, and often as long as thirty to forty minutes.

As the experiments were not of a quantitative nature no effort was made to determine the limits of the activities; we merely desired to expose the organisms for such time and under such circumstances as would insure the survival of some of them. We hoped especially to demonstrate the influence of such environment on the functional activities of the survivors and their offspring.

At the termination of the time set for the exposures, three or four drops of the mixture of organisms and germicide were transferred by means of a dropping pipette to about ten or twelve cubic centimeters of normal nutrient bouillon and, after thorough mixing, three or four large wire loopfuls from this were transferred to the surfaces of several tubes of slanted normal nutrient agar, and kept at body temperature until the surviving cocci had developed into colonies.

The routine followed was briefly: to expose the organism, as noted above, to the chemical chosen; as soon as the survivors had fully developed into colonies, to expose these in turn by the same method; and to continue this through a long series of exposures studying as we progressed the general characteristics of the survivors.

By following this plan we succeeded in making forty-four successive exposures to 1 : 1,000 corrosive sublimate in the first, and fifty-six in the second series.

To phenol solution we made sixty-four successive exposures; while to the saturated sodium carbonate solution twenty-five exposures were made. All could have been carried farther, but as nothing apparently could have been gained we discontinued them at these points.

Results.—In all cases the first or primary exposures were of necessity of but brief duration, sometimes less than five

minutes, except in the case of the sodium carbonate mixtures, which could always be very much longer.

After a few exposures, however, greater resistance was manifested, and the time of exposure could be extended rapidly, practically always with a fair number of surviving colonies, though occasionally only two or three would develop on the normal test medium.

This gradual increase in resistance or tolerance has, however, its limits, and when the maximum is reached a very little longer exposure often results in total destruction, so that it becomes necessary to recede in the series and begin again.

In the case of survivors after the exposure to sublimate, their colonies rarely develop to a point visible to the naked eye sooner than forty-eight hours at incubator temperature; while in the case of those surviving exposure to phenol and to sodium carbonate, colony development was frequently manifest in eighteen hours after their transfer to normal media, at body temperature.

Functional changes.—Under the circumstances of our experiment chromogenesis is the first function to manifest modification. After ten or fifteen successive exposures (the advent of the phenomenon varies) the colonies developing from the surviving cocci are more brilliantly orange in color than is the case with *staphylococcus* cultures, taken at random from laboratory stock or secured from suppurating lesions in man or animals.

This change is particularly noticeable in the survivors after the exposure to sublimate and phenol. It is less conspicuous among those organisms that survive the action of sodium carbonate. It is interesting to note, moreover, that this color accentuation characterizes not only surviving colonies themselves, but their offspring to the eighth and twelfth generations on normal culture media. In one series I have carried the subcultivation to the twentieth generation (with twenty-four-hour intervals) on normal agar agar at body temperature. In this case there is a developing

tendency to reversion, that is, the later generations show less and less color production. This tendency to revert, in so far as this phenomenon goes, is least conspicuous among the survivors of the phenol series. Under all circumstances the color production is greater at room temperature and diffuse light than in the incubator, though the immediate survivors from both sublimate and phenol exposures are, as a rule, brilliantly orange in color immediately upon being taken from the incubator, when the colonies are fully developed (that is, ranging in size from .5 millimeter to 2 millimeters in diameter).

The next interesting functional modification resulting from the exposures is a pronounced increase in the power of the surviving colonies to liquefy gelatine.

If strains of *Staphylococcus pyogenes aureus* be selected in which this function is low, repeated exposures to either mercuric chloride, phenol or sodium carbonate result in a gradual increase in the intensity of this function. Beginning with a variety that has the minimum power to liquefy gelatine, we find, in the case of sublimate exposures, that after the tenth or twelfth successive exposure an increase is noted, and after the fortieth or fiftieth successive exposure this function is so accentuated that liquefaction progresses at approximately one hundred fold the rate noted at the beginning of the experiment. The same is seen after exposure to phenol and to a less extent after an exposure to sodium carbonate.

The maximum of proteolytic power as seen in the immediate surviving colonies is not permanent; for instance, if after the fortieth or fiftieth exposure one begins a series of subcultures from the surviving colonies using normal nutrient agar agar at body temperature, it is found that at or about the tenth subculture there is a loss of approximately fifty per cent in the rate of liquefaction, but if with twenty-four-hour intervals this subcultivation be continued there is, up to the twentieth subculture, no appreciable further diminution in the activity of this function. Even such a reduction as fifty

per cent in the observed maximum rate still leaves a degree of activity in this function that is in striking contrast with that noted in the culture with which the series was begun.

In the course of this study, repeated efforts were made to determine if the colonies surviving the exposures to the germicides evinced any degree of modification in their sugar splitting activities or in their power to clot milk. Up to the present time such modification has not been observed.

Thus far our method seems to have resulted in the gradual selection from among the mass of cocci with which we began the most resistant, therefore the most characteristic, individuals, and in large measure I am disposed to think this the case, but subsequent observations have led to some degree of confusion in this connection, so that the general interpretation is not so obvious.

The interesting and important demonstrations that have been made, especially by Altmann and his associates,² on the loss of complement deflecting power experienced by certain bacteria when continuously cultivated upon media containing germicides, led us to investigate the properties of our exposed cocci not on complement deflection, but rather on the less complicated phenomenon of agglutination. Since staphylococci naturally clump, as seen under the microscope, the macroscopic method of making the agglutination test was used. For this we employed very narrow test-tubes, having a little over one cubic centimeter capacity. These were filled to the one cubic centimeter mark with mixtures of cocci, suspended in sodium chloride solution (.85 per cent) and immune sera in such proportions as to expose the cocci to the desired dilutions of serum, namely, 1 : 300; 1 : 600, and 1 : 1,000. After such mixtures had stood in the incubator for two hours they were placed in the ice chest, and the results noted after about eighteen hours. Briefly stated, these were as follows :

The serum of rabbits that had received three or four intraperitoneal injections of normal *Staphylococcus aureus* that had been killed by exposure to heat, not exceeding 60° C.,

caused a complete precipitation of all suspended normal cocci in 1 : 1,000 dilution; the supernatant fluid in the tubes being quite clear, colorless and transparent as compared with the cloudy control tube, and having at its bottom a flocculent precipitate of agglutinated cocci. On the other hand, no such agglutination occurred with the cultures that had survived the twentieth and forty-fifth exposures to corrosive sublimate and the forty-seventh and fifty-sixth exposure to phenol even in such low dilutions as 1 : 300. Survivors from the seventeenth successive exposure to saturated sodium carbonate solution also failed to agglutinate with 1 : 300 dilution.

With the hope of securing an explanation for this phenomenon, a second series of rabbits was immunized with cultures that had survived repeated exposures to both corrosive sublimate and phenol; these, as just noted, being varieties that fail to agglutinate with the serum of animals immune from normal staphylococci. In one of these series, the survivors had had from nineteen to twenty-four successive exposures to corrosive sublimate, while in another the rabbits were immunized with cocci that had survived from forty-nine to fifty-three exposures to phenol in the strength given above. The method of immunization was the same as that used with the normal cocci. The results are both interesting and puzzling, but not very satisfactory in the way of offering an obvious explanation of the phenomenon under consideration. They are briefly:

Rabbits immunized with staphylococci that have survived repeated exposures to both corrosive sublimate and phenol supply sera rich in an agglutinating principle that reacts positively and in as high as 1 : 1,000 dilution on normal *Staphylococci aureus*, but do not agglutinate the strains with which the animals were immunized, even in so high a concentration as one part of serum to three hundred parts of salt solution, that is to say, the organisms that have survived the exposures to sublimate and phenol have lost their power to agglutinate in the above mentioned dilutions, but have retained the power to call forth agglutinin production in the bodies of animals into which they may be injected. In such

concentration as one serum to ten or twenty salt solution both normal and exposed cocci are agglutinated.

I am not sure that a satisfactory explanation for this is forthcoming. Two may be offered :

The surviving organisms used in the test may have failed to agglutinate because of the presence of the chemicals superficially deposited upon them, thus interrupting the interaction between their surfaces and the agglutinin in the serum, or the chemicals may have destroyed the superficial receptors of the cocci without such destructive action having penetrated so deeply into the bodies of the cocci as to render inactive all specific antigens.

If either of these be the correct explanation we should find that cultivation of the non-agglutinating strains upon normal media, without again exposing them to the hurtful germicides would result, after a while, in supplying descendants free of all adherent chemicals, if the first explanation be correct or, if not, there might be, under these favorable conditions, a regeneration of the surface receptors that are presumed, in the second explanation, to have been destroyed or otherwise rendered inactive. As a matter of fact we find that after the fifth or sixth generation on normal media under the usual favorable conditions of growth the exposed strains of cocci gradually regain their power to agglutinate, and ultimately are indistinguishable in this respect from staphylococci that had never been exposed to germicides.

Pathogenesis. — Several attempts have been made to detect an influence of such exposures to germicides as I here mention upon the pathogenic properties of the several strains of *Staphylococcus aureus* upon which we have been working, but the results do not justify the opinion that this function is appreciably affected.

We present these results because they throw some light upon the question of variation. In the routine work of every laboratory, functional variations among definite species of bacteria are constantly being encountered. For example,

we have at present four strains of staphylococci that came from suppurative processes in man that grow with more or less orange color and cause typical abscesses and fatal septicemia in rabbits after intravenous injection. All except one have been passed through the bodies of rabbits, yet two of them liquefy gelatine scarcely, if at all; one does this only slowly, while the fourth, recently isolated from man, has this function developed to a conspicuous degree. Their action upon other media is likewise without uniformity, yet there can be no doubt that they are the ubiquitous *Staphylococcus pyogenes aureus*, even though they differ in certain important features the one from the other and from the type organism as ordinarily described in the books.

While it is a simple matter to reach this conclusion, it is far from simple to identify the environment influences that have caused the functional fluctuation that differentiates some of these anomalies from that which we regard as normal, nor does it now seem to us easy to decide precisely what shall be called normal, at least in so far as the organisms with which we have occupied ourselves are concerned.

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ON THE METAPLASIA OF BRONCHIAL EPITHELIUM.*

SAMUEL R. HAYTHORN, M.D.

(Assistant Professor of Pathology, University of Pittsburgh, Pittsburgh, Pa.)

During the study of a case of unresolved pneumonia, which contained both areas of organization and abscesses, one field was found which contained two medium-sized bronchi, the mucosa of which was replaced by granulation tissue covered by stratified squamous epithelium. The region from which this block came was then more carefully searched and as many as twelve bronchi were found which showed a like condition. Similar metaplasias of the bronchial epithelium have been recorded from time to time, but this was an unusually good example, on account of its wide extent and owing to the fact that, among the squamous cells, islands of columnar cells remained.

The term "metaplasia" was introduced by Virchow about 1854 and was defined by him as the direct change of one cell into another cell which was architecturally different but which had not completely given up its characteristics or structure.

Schridde holds that this view is untenable, as no facts to support it have been observed either in intra- or extra- uterine life. He designates it "direct metaplasia" as opposed to his own theory of "indirect metaplasia." He believes that the formative cells of the growing layers of any kind of epithelium, or their immediate daughter cells, may give up their specific attributes and revert to a cell which has all the powers of differentiation possessed by the embryonic cells from which the epithelium developed. In other words, such cells may form any type of epithelium, but their power of differentiation is limited to the formation of the epithelial tissues and does not include the transformation into connective tissue or endothelium. Schridde states that by the atypical differentiation of such cells a kind of epithelium

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may be formed which is "heterotype" for the region in which it occurs, and cites as an example the occasional presence of squamous celled plaques in the cardiac end of the stomach.

Lubarsch in general accepts Schridde's view but believes further that completely differentiated cells may undergo the change.

He divides metaplasia into (1) pseudo metaplasia; (2) retrograde differentiation of cells, including Hanseman's anaplasia; and (3) real metaplasia such as is seen in squamous celled tumors of the stomach and prostate.

The author did not have access to the original articles of Lubarsch and Schridde, but by the term "pseudo metaplasia" understands the direct extension of one type of epithelium into a region normally covered by another type, *e.g.*, "ectropion of the cervix," and squamous celled extension to the middle ear after paracentesis. Hansemann's anaplasia is described as a cell change in which there is a loss of a number of chromosomes bringing about an increased power of growth at the expense of cell function.

Ribbert is opposed to the whole idea of metaplasia and explains the changes as coming from: (a) cells which have been actually misplaced during embryonic life; (b) cells which may appear normal for the region in which they occur but which possess embryonic potentialities; and (c) cells which are only partly differentiated and which undergo changes more readily. He thinks that a certain combination of circumstances must also be present before such a change can occur, and for this influence lays stress upon the effects of surrounding supportive tissues. He further points out that newly developed cells are never as highly specialized as the preëxisting cells.

Many other views have been brought forward, but the principal points of the discussion are covered by these four observers.

In discussing the case in hand a few points concerning the normal histology of the part may well be reviewed. Kölliker describes three layers of epithelial cells in bronchi

large enough to possess cartilaginous plaques; an outer layer of ciliated and goblet cells, a middle layer of somewhat spindle-shaped replacing cells, and an inner layer of pyramidal formative cells, which rests upon a so-called hyaline membrana propria or basement membrane. Bronchi of less than one millimeter diameter have only a single layer of cells and a basement membrane difficult of demonstration.

The bronchial lesions in the case we are considering were varied. Some sections contained large inflamed bronchi with granulation tissue in the mucosa and a complete denudation of all types of epithelium. The lumina were filled with leucocytes and cellular débris. Other sections showed a thick unbroken stratified squamous epithelium lining the bronchi. These epithelial membranes were usually slightly raised from the granulations by a serous exudate beneath them. Small nests of ciliated columnar cells remained in some of the crypt-like depressions of the mucosa in areas where most of the surface had a squamous cell covering. At the points of the junction of the two types of cells the squamous layers seemed to be continuous with the formative layers of the columnar cell portions.

In still other areas typical ciliated columnar cells were found resting on top of a double layer of low cuboidal cells and appeared to be almost ready to desquamate. If this desquamation had occurred no trace of columnar cells would have remained on the surface. In none of the involved bronchi were the basement membranes demonstrable.

In the lung tissue proper there were signs of a rather prolonged inflammation; larger and smaller abscesses were present; the fibrin had the appearance of being old and hyaline, and in many places actual organization had taken place. Some of the alveoli were free from fibrin and instead contained large amounts of serous fluid and many pigment-bearing endothelial cells. The alveolar walls were thickened and the pleura was much fibrosed.

In seeking an explanation for the change from columnar to squamous cells each layer mentioned above must be considered.

First, the ciliated and goblet cell layer was found remaining only in the most protected depressions and was never seen to be in any way connected with the changed cells. On the contrary, where it was not already destroyed, it was being thrown off into the lumen. From this it seemed that there was no evidence that the direct metaplasia of Virchow, the change of the columnar ciliated to the squamous cell, had occurred.

In the "replacing layer" one could not feel so certain, for no cells resembling them were found. It was probable that they had either desquamated directly or had become ciliated and then desquamated. The extensive granulations in the mucosa would point to a wide desquamation of epithelium having taken place. The regeneration must have come, then, from certain remaining cells of the growing layer, and it is to these cells that we looked for evidence of the changed progeny. These cells themselves showed little further than that they were more polygonal and less pyramidal than normal. But their environment was changed, for the basement membrane had disappeared and they rested directly upon granulation tissue. This fact seemed very important, for partial desquamation followed by complete regeneration we believe is a common occurrence in acute inflammation of the bronchi.

In diphtheria, fibrin casts including the epithelial lining of the bronchi may be coughed up, and here Mallory has pointed out the infrequency with which one sees the diphtheritic exudate extending below the membrana propria. Its action is believed to be that of a limiting membrane which allows the separation of the cast to take place. It also serves as a favorable surface for the regeneration of the normal columnar cells.

Three other cases of metaplasia of bronchial epithelium were studied. One was a case of broncho-pneumonia with abscesses. Instances were observed where bronchi, whose walls were partially destroyed by abscesses, retained an uninjured basement membrane in other less severely attacked parts. In one bronchus, where the membrane was broken

only at one point, there was present at that point an island of squamous cells. In the second case a large secondary growth of sarcomatous nature had injured many bronchi and partial regeneration had occurred. Stratified squamous epithelium had replaced the normal columnar cells in some of the bronchi, and definite basement membranes were not demonstrable.

The third case was a primary carcinoma of the lung arising in the bronchi. Here the normal relations were completely lost. The lower layer of cells remained columnar in shape, while the more superficial layers were of typical stratified squamous cells.

In none of the cases was there any example of stratified squamous epithelium resting on a well developed basement membrane.

Difficulty was experienced in demonstrating a definite basement membrane in some apparently normal bronchi from other cases, and the membranes when present did not always react equally toward the special stains generally recommended for demonstrating them. Their histological appearance and chemical nature did not seem to be constant for bronchi of any given size. This latter observation is mentioned as an important obstacle in the way of proving that the destruction or absence of the membrana propria is the essential factor in epithelial metaplasia of the bronchi. A list of stains used is given in the order of their efficiency: Mallory's phosphotungstic acid hematoxylin; Pianese's picronigrosin; Van Giesen's connective tissue; Mallory's aniline blue; hematoxylin-eosin; eosin methylene blue. The membrana propria did not react to Verhoeff's or Weigert's elastic stains as stated in some texts.

In connection with the study of these cases, Dr. Klotz called my attention to a good example of metaplasia of the stratified squamous type in the pancreatic ducts. The ducts were dilated and some contained calculi, while others were almost completely filled with the proliferated squamous cells. The remainder of the pancreas showed rather advanced chronic interstitial pancreatitis. In the affected ducts the

walls were actually inflamed. Many polynuclear leucocytes were present. As the basement membrane of the pancreatic duct is not comparable to that of the lung, the case, while interesting, does not bear directly on the relation, if indeed any exists, between metaplasia and the presence or absence of the membrana propria.

CONCLUSIONS.

1. The evidence concerning metaplasia, as observed in this case, is strongly against the theory of the "direct metaplasia" of Virchow.

2. Our findings, in a measure, agree with Schridde's idea in so far as they show that the metaplastic cells are newly formed cells and that they come from the growing layer.

3. They support Ribbert's views in that the new-formed cells are less highly specialized than the normal cells, and also that the surrounding tissue changes have a strong influence on the type of the new cells formed.

4. They seem to contradict the necessity of the presence of embryonic rests, as they could hardly have been so numerous as simultaneously to set up the process in several different bronchi.

5. Another idea which is brought forward is that the bronchial mucosa destroyed beyond the hope of specific regeneration seeks to protect itself with the best reparative cells it can produce in its exhaustive state.

6. Assuming that there is a histo-chemical identity of the so-called basement membrane, it suggests that there is a link between the injury to these membranes and tumor growth; that is, that the irritants and inflammations which destroy them open the way for metaplasia and new growth.

[Thanks are due Dr. Klotz for his valuable suggestions during the preparation of this paper.]

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DESCRIPTION OF PLATES.

PLATE XXX.

FIG. 1. — Squamous cell metaplasia of bronchial epithelium. L.P.

FIG. 2 — Squamous cell metaplasia of one bronchus, columnar cells remaining in adjacent bronchial epithelium. L.P.

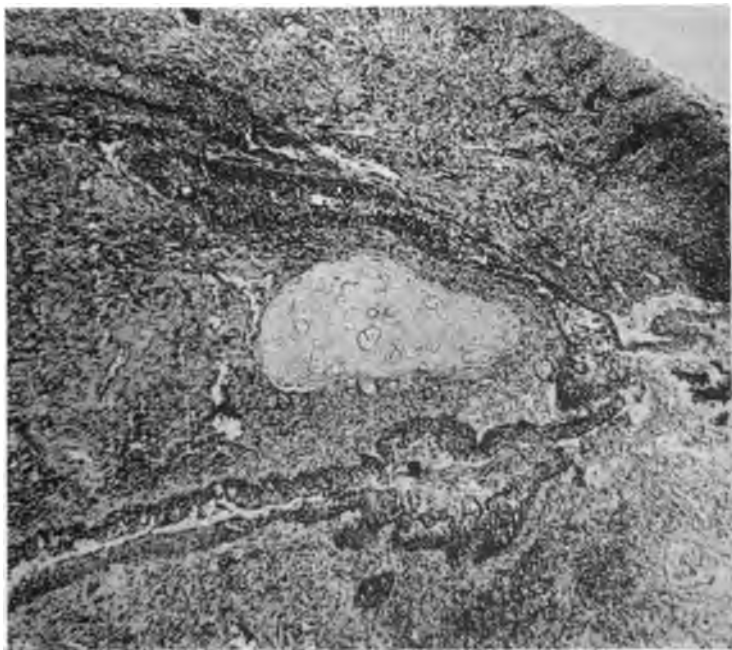
PLATE XXXI.

FIG. 3. — Squamous cell metaplasia of bronchial epithelium showing a crypt covered with columnar cells. The basal layer is continuous for both columnar and squamous types. H.P.

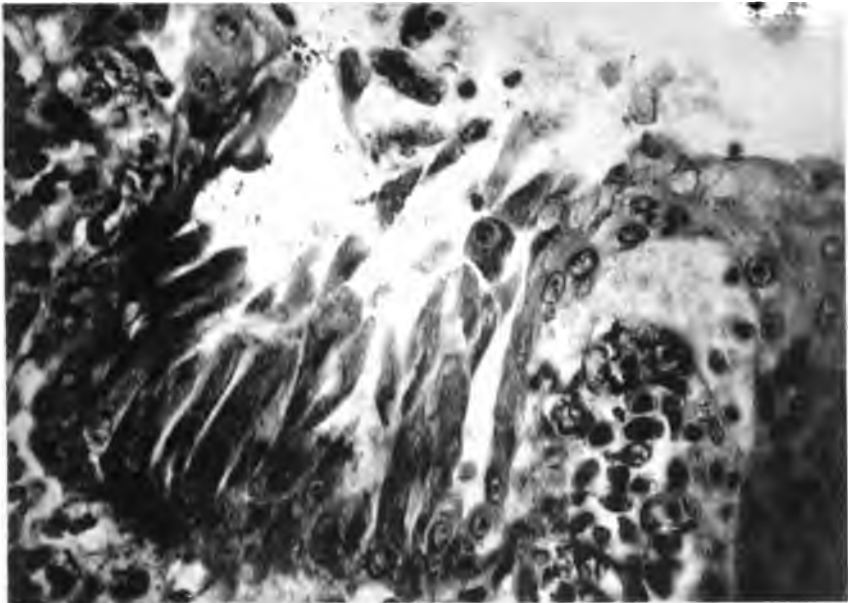
FIG. 4. — Squamous cell metaplasia of bronchial epithelium. Crypt showing a layer of columnar cells resting on a double layer of cuboidal cells. Mass of cells to the right of squamous type.



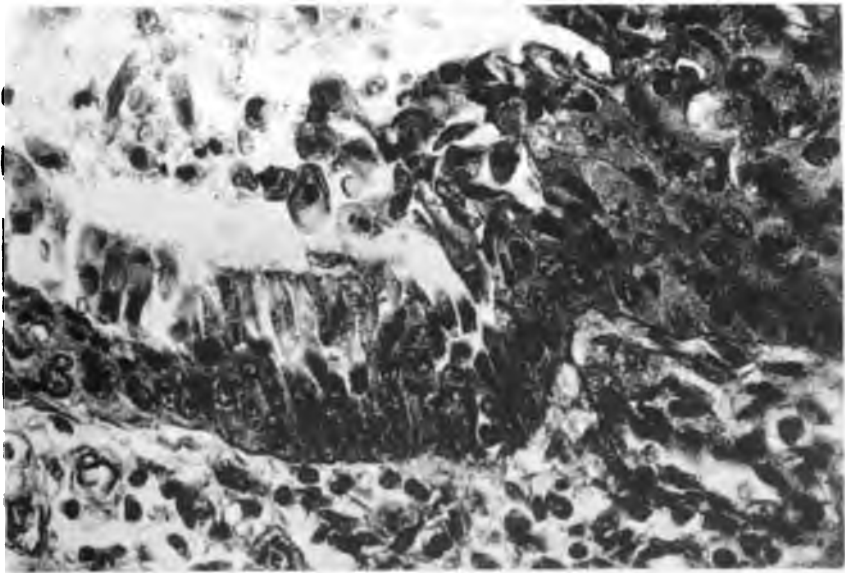
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DETERMINATION OF THE CHOLESTEROL CONTENT OF HUMAN SERUM BY THE COLORIMETRIC METHOD.*

PAUL G. WESTON AND GRACE HELEN KENT.

(*From the Pathological Laboratory, State Hospital for the Insane, Warren, Pa.*)

In estimating the cholesterol content of human serum we have employed the method which has already been described by one of us.¹

The application of this method to a particular problem has not suggested any essential modifications of the method itself; but in the course of the experiments upon the cholesterol contained in serum some additional data have been obtained concerning the reaction of pure cholesterol. It seems advisable, therefore, to open this paper with a supplementary description of the conditions under which the method has been found to give the most satisfactory results.

1. Application of the colorimetric method. — The most serious difficulty which we have encountered has been presented by the instability of the characteristic pink color. It is essential that the comparison of unknown solutions with known be made at the time when the differences are most apparent. The addition of the second cubic centimeter of chloroform entirely obliterates the color of the solutions, so that they all appear alike. The pink color then develops gradually and reaches its greatest depth in from fifteen to thirty minutes. After this there is a gradual transition from pink to violet, which finally fades to a pale gray. In the last stage as in the first the solutions of different strength appear very nearly alike, and this similarity begins to appear in the violet stage. The time when the solutions can be differentiated with the greatest accuracy is immediately after the pink color has reached its greatest depth and before it begins to show a trace of violet. The duration of this particular stage varies greatly; under some conditions the violet appears

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before the pink has fully developed, and in this case no comparisons are possible. We have found it necessary, therefore, to make a special study of the external conditions of the experiment in order to determine by what means the reaction may be sufficiently retarded to allow ample time for observation at the most favorable stage.

The rapidity of the reaction depends in part upon the strength of the sulphuric acid. If acid of 1.84 specific gravity is used the reaction takes place too rapidly and the violet color may develop immediately after the second cubic centimeter of chloroform is added. We have found it advantageous to use acid which has been exposed to the air sufficiently to reduce its specific gravity to 1.81; acid thus prepared has given very satisfactory results.

The reaction may be retarded by placing the tubes in a dark place. Repeated experiments have shown that protection from the light for at least fifteen minutes after the addition of the second cubic centimeter of chloroform is favorable to a stable pink color. If the solutions are exposed to strong sunlight during this period they turn violet before any observations can be made.

These experiments were made in winter at a room temperature of about 26.5° C. In any given test we have been careful to keep all conditions uniform for all the solutions which were to be compared, but we have not found it necessary to keep them at any given temperature. A few experiments have been made for the purpose of determining the effect of temperature upon the reaction; the results indicate that ordinary variations are negligible but that both extremes are unfavorable.

Each tube must be thoroughly shaken immediately after the sulphuric acid is added to it. In order to insure uniformity in handling the tubes we have regularly followed a definite plan: one of us added the acid, dropping it into the tube with a pipette, and the other immediately took the tube between the thumb and finger and gave it twenty shakes, the thumb being protected from the acid by a rubber glove finger. When the second cubic centimeter of chloroform

was added the tubes were not disturbed in the rack, but care was taken to allow the chloroform to run directly from the burette into the bottom of the tube.

While this series of tests was in progress there was a period of several weeks during which we were unable to obtain Merck's chloroform, and we therefore had occasion to try various chloroforms of domestic manufacture. One particular lot gave fair results, but for the most part the results were hopelessly unsatisfactory, and in many cases the characteristic pink color entirely failed to appear. After much experimentation we learned that inferior chloroform which answered to the usual tests for purity could be greatly improved by being washed with water and then distilled. By treating it thus we were able to make some use of it. We have found also that chloroform which has been used in these tests can be so prepared as to be fit for further use. After making the observations upon a set of tubes, we decant the chloroform and preserve it; this chloroform must be distilled, shaken up with water, and again distilled, after which it may be regularly used for the additional cubic centimeter, but not for the chloroform in which the cholesterol is first dissolved. The original solution must be made from fresh chloroform, preferably Merck's blue label. For the second cubic centimeter, chloroform which has been used gives better results than new chloroform which has not been washed. Even when depending wholly upon Merck's chloroform we have sometimes found it necessary to wash it with distilled water before using it for the second cubic centimeter.

We have found it worth while to give the most careful attention to the details of the technic. The test-tubes were sorted out according to size, and in each test the tubes of a given size were used for both the known and the unknown solutions. The inside diameter of the preferred tubes was nine millimeters. Measurement of the tubes with calipers proved to be unsatisfactory, and we found it necessary to pipette a given amount of water into each tube, and then arrange the tubes according to the height of the water.

Throughout the experiment we have used certified glassware, and we have greatly reduced inaccuracies by making up all solutions in relatively large quantities. One-half gram of pure cholesterol was dissolved in fifty cubic centimeters of chloroform, and any desired quantity was taken with the pipette from this solution, the chloroform being evaporated if necessary. We have kept regularly on hand nine standard solutions with which all unknown quantities of cholesterol were compared; these solutions (.0001, .000125, .00015, .000175, .0002, .000225, .00025, .000275, and .0003 gram of cholesterol to one cubic centimeter of chloroform) were prepared in quantities of not less than ten cubic centimeters, a solution of .0005 gram of cholesterol to one cubic centimeter of chloroform being used as the basis for dilution.

We have attempted to prepare a series of permanent standards corresponding in color to these solutions at the time when the pink color is at its greatest depth. Various inks and aniline dyes were used for this purpose. The colors of the cholesterol solutions were easily matched, but the artificial colors soon faded, and we have not been able to find any suitable coloring matter which will bear exposure to the light. A series of standard colors, if permanent, would be useful for the preliminary tests which are made for the purpose of determining what dilution is necessary in order to bring an unknown solution within the range of the known series. But it could hardly be used for accurate measurements; the color of the cholesterol solutions, even when they are prepared under the most favorable conditions, undergoes a perceptible change while the observations are being made, and it is essential that the changes be uniform in the known and unknown solutions.

2. Extraction of cholesterol from serum. — We have not been able in a single instance to isolate pure cholesterol from serum. We have been concerned mainly with small quantities of serum, too small to yield conveniently weighable quantities of cholesterol; but in order to test the colorimetric method we attempted to make determinations upon

a few samples of serum both by weight and by colorimetric estimation. With this in view we extracted one sample of eighty cubic centimeters and two smaller samples by the method used by Koch for the extraction of cholesterol from brain tissue.³ In each case the resulting product was an oily substance of a deep yellow color. We did not attempt to use Ritter's method.⁸

For colorimetric determinations it is not necessary that the yield of cholesterol should be pure. All impurities which do not affect the color of the reaction may be disregarded. As there is no method known to us by which all the cholesterol can be extracted from body fluids in a pure state, we abandoned the attempt to isolate it pure, and directed our attention toward the development of a method of extraction which should be complete, but as simple and economical as possible.

For the determination of the cholesterol content of an individual sample of serum we recommend the following method:

Two and one-half cubic centimeters of serum is pipetted into a wide mouthed, glass stoppered bottle of thirty cubic centimeters capacity. Twenty cubic centimeters of alcohol is added, the bottle being gently shaken as the alcohol is poured in. This is kept at a temperature of 60° C. for at least one day, then the extract is decanted into a flask and the residue covered with ether. This is allowed to stand for at least one day, after which the ether extract is added to the alcohol extract. The residue is washed three times in boiling alcohol, the extract being decanted or filtered into the flask. The total amount of alcohol used, including the first extract, is about sixty or seventy cubic centimeters. A small amount of sodium hydrate is added to the extract, roughly about .25 gram. It is then allowed to boil slowly over a hot plate for two hours, during which time it should evaporate to about ten cubic centimeters. Fifty cubic centimeters of saturated solution of calcium hydrate in distilled water is added. This forms a light flocculent precipitate which, when it is allowed to collect, leaves the fluid perfectly clear; this precipitate

includes the cholesterol. The extract is filtered, and the residue is returned, on the paper, to the flask. The lip of the flask is carefully wiped with a scrap of moist filter paper, and this also is thrown in. The flask is allowed to stand until the paper is entirely dry, after which the cholesterol is extracted with ether and decanted or filtered into another flask. The first flask must be washed four times with ether, and it requires in all at least one hundred cubic centimeters. The ether in the second flask is evaporated, and the flask is dried and cooled. Five cubic centimeters of chloroform (Merck's blue label) is added, two cubic centimeters of chloroform being allowed for each cubic centimeter of serum. The chloroform extract is filtered into a vial, and is now ready to be tested.

The cholesterol content is estimated by comparing the reaction obtained from a dilution of this extract with that obtained from the nine standard solutions of pure cholesterol. The solutions are prepared in test-tubes, and when all are ready .1 cubic centimeter of sulphuric acid is dropped into each tube, according to the method described above. Thirty minutes later one cubic centimeter of chloroform is added to each tube, after which they are placed in the dark for fifteen minutes. From this time they should be watched carefully; when the standard solutions show a perfect and distinct gradation from a barely perceptible pink to a deep pink it is time to compare the colors and to record the equivalent in the known series of each unknown solution. One set of the standard solutions may serve for as many as twenty or thirty individual tests, provided that all are prepared at the same time and in test-tubes of the same size.

In order to balance the inevitable errors in pipetting, every specimen of serum should be tested several times. When a given serum is tested for the first time it is well to use .2 cubic centimeter of the extract to .8 cubic centimeter of chloroform. This will probably fall within the range of the standard solutions. In subsequent tests the serum extract should be so diluted as to bring it near or slightly below the middle of the scale. The quantities most

commonly used are .15, .2, and .25 cubic centimeter of the extract, representing, respectively, .075, .1, and .125 cubic centimeter of serum.

Both in the extraction of the serum and in the estimation of the cholesterol there are numerous sources of error. In order to insure that the extraction is complete it is well to test all residues qualitatively by the Salkowski or the Liebermann test. The serum coagulum, after it has received four washings in hot alcohol, may be allowed to stand for several days in ether or hot alcohol; this is collected in a test-tube and evaporated, then redissolved in chloroform and tested. The filtrate from the calcium hydrate precipitate may be evaporated, and the residue dissolved in chloroform and tested. Finally, when the extract is transferred from the first flask to the second, the first flask may be given an additional washing in ether for the purpose of testing it. In all extractions by methods not perfectly familiar to us we have made a practice of testing all residues theoretically free from cholesterol, and have discarded all methods of extraction thus shown to be imperfect.

After the extract is in the second flask there are possibilities of error in either direction. A trifling inaccuracy in the measurement of the chloroform which is added may cause a measurable error in the estimate of the cholesterol. If two independently extracted samples of the same serum give different results, the natural inference is that the larger is the more nearly correct. This, however, is not necessarily the case; in one instance our smaller estimate was confirmed by twelve independent extractions, and we were forced to the conclusion that the larger estimate was incorrect.

In the extraction of the serum, errors on the positive side are exceptional. But in the measurement of the cholesterol contained in a given extract, positive errors are more frequent than negative ones; in pipetting the chloroform extract into the test-tube it is the excessive quantity that must be especially guarded against rather than the insufficient quantity. Error from this source could probably be reduced by making up the extract with a larger proportion

of chloroform than was used in these experiments (two cubic centimeters of chloroform to one of serum).

In developing a method of extraction we have used the solvents, acetone, ether, alcohol, and acetic ether, in various combinations and for varying periods of time. The results of the preliminary experiments are hardly worthy of being stated in full, and it seems better to summarize briefly the conclusions drawn from them.

1. If ether is to be used successfully as a solvent the serum should first be precipitated in alcohol or acetone.

2. If the extraction is to be made with cold reagents, considerable time may be saved by keeping the serum at a temperature of 60° C. for one hour, before adding any reagent. This preliminary heating of the serum is not necessary if it is to be extracted by hot alcohol.

3. The presence of fatty acids in the serum extract interferes with the reaction, and it is necessary to saponify them.

4. If acetic ether is to be used in the extraction it must be evaporated before the sodium hydrate is added.

5. The serum may be kept for a week or more either at room or ice-box temperature and with or without aseptic precautions; the yield of cholesterol will not be affected by these conditions.

The method which we recommend is the simplest one which has invariably given satisfactory results. Amongst the various methods we have tried, using different samples of the same serum, the following may be cited:

1. Alcohol at 60° C. for one day, ether for one day, as described above.

Estimate, per cubic centimeter of serum, three milligrams.

2. Alcohol at 60° C. for five days, ether for two days.

Estimate, per cubic centimeter of serum, 2.58 milligrams.

3. Precipitated in acetone, covered with ether and allowed to stand for one day, alcohol at 60° C. for one hour.

Estimate, per cubic centimeter of serum, three milligrams.

4. Precipitated in acetone, covered with ether and allowed to stand for three days, alcohol at 60° C. for two days.

Estimate, per cubic centimeter of serum, 2.7 milligrams.

5. Alcohol at 60° C. for five days.

Estimate, per cubic centimeter of serum, 3 milligrams.

6. Alcohol at 60° C. for four days, acetic ether for two days; extract evaporated to dryness, more alcohol added for saponification.

Estimate, per cubic centimeter of serum, 2.66 milligrams.

It seems probable that the variations in these results are accidental, rather than that they indicate a positive advantage of some of the methods used over others.

In all the instances cited the serum coagulum was thoroughly washed in alcohol and the fatty acids were saponified by boiling with sodium hydrate for two hours. We have not determined the minimal quantity of sodium hydrate necessary to effect the desired reaction, nor the exact length of time required for boiling. One-tenth gram of sodium hydrate to one cubic centimeter of serum, boiled for two hours, has given satisfactory results; it is possible that a much smaller quantity would be sufficient.

In order to determine the effect of this treatment upon the quantity of cholesterol we dissolved pure cholesterol in alcohol, added sodium hydrate and boiled for two hours, recovered the cholesterol with ether, dissolved it in chloroform and estimated the amount by comparison with the standard solutions. The results indicated that no cholesterol was lost.

Instead of precipitating the soap with calcium hydrate it is possible to evaporate the extract almost to dryness and then extract the cholesterol with ether. But it is difficult, on account of the presence of glycerine, to dry the flask; we have found it more convenient therefore to precipitate the soap with calcium hydrate and then filter off the glycerine. We have made a practice of testing the filtrate for cholesterol, but have never obtained the faintest trace of a reaction. We have tested the method further by extracting

two samples of the same serum by the same method up to the conclusion of the saponification, after which one was extracted with ether from the dried flask, while the other was precipitated with calcium hydrate and filtered. The quantitative estimates obtained from the two extracts were identical.

3. Recovery of pure cholesterol added to serum. — In order to establish the validity of the method of extraction we added pure cholesterol to six samples of serum, one milligram being allowed for each cubic centimeter of serum; these were extracted, side by side with normal samples of the same sera, by the method recommended. The treated specimens were prepared in the following manner: .25 cubic centimeter of the stock solution containing .01 gram of cholesterol to one cubic centimeter of chloroform was measured into a bottle, and the chloroform was evaporated; a small quantity of ether was poured into the bottle and, while the cholesterol was in solution in the ether, 2.5 cubic centimeters of serum were added; as the ether evaporated crystals appeared on the surface; the bottle was shaken and was allowed to stand open for several hours, after which alcohol was added and the extraction was carried on as usual. The results of this experiment are shown in Table I. Seven estimates were made upon each extract, and the results of the seven observations are given in full, followed by the average.

TABLE I.
(*Milligrams per cubic centimeter.*)

	Individual Estimates.							Average.
Serum 1	1.6	1.66	1.75	1.66	1.75	2	1.75	1.74
1 + 1 milligram	2.75	2.66	2.75	3	2.75	3	3	2.84
Serum 2	2	2.25	2.33	2	2	2.33	2.33	2.18
2 + 1 milligram	3.5	3.33	3.33	3	3	3	3	3.16
Serum 3 a	1.6	1.5	1.5	1.5	1.6	1.5	1.5	1.53
Serum 3 b	1.75	1.6	1.66	1.5	1.5	1.6	1.75	1.62
3 + 1 (?) milligram	2.66	3	3	3	3	3	3	2.95
Serum 4	1.2	1.5	1.75	1.6	1.5	1.5	1.6	1.52
4 + 1 milligram	2.5	2.5	2.66	2.5	2.5	2.33	2.66	2.52
Serum 5	1.2	1.5	1.5	1.6	1.6	1.5	1.5	1.48
5 + 1 milligram	2	2.5	2.33	2.33	2.33	2.5	2.5	2.35
Serum 6	1.2	1.25	1.5	1.6	1.5	1.5	1.6	1.45
6 + 1 milligram	2	2.5	2.33	2.33	2.5	2.33	2.5	2.34

In the case of Serum 3 there is evidently an error; the estimate shows an increase in the treated specimen of more than one milligram to the cubic centimeter of serum, and it is plain either that more than 2.5 milligrams of cholesterol were added to this specimen or else that the normal specimens were imperfectly extracted. As we had some of the serum on hand at the time when the discrepancy was discovered we extracted a second normal sample and the estimates made upon the two extracts are given separately. If the larger estimate is accepted the treated specimen shows an excess of .33 milligram of cholesterol to the cubic centimeter of serum. We think it probable that .35 cubic centimeter of the cholesterol solution was pipetted into the bottle instead of .25 cubic centimeter. This would fully account for the excess and the treated specimen would in this case show a loss of .07 milligram to the cubic centimeter.

4. Estimates obtained from individual sera. — The serum used in this investigation was obtained from various sources. Many attendants employed in this institution voluntarily contributed from ten to two hundred and fifty cubic centimeters of blood. While the experiment was in progress the sera of over sixty patients were tested for the Wassermann reaction, and any serum remaining after the Wassermann test had been made was used in these experiments.

The estimates of the cholesterol content of fifty-two sera are given in Table II. Each estimate represents the average of all the observations. In order to increase the number of individual determinations we included in this table the estimates obtained from the sera of twenty-four insane patients. All the subjects were males except Nos. 21, 28, 48, 51, and 52.

TABLE II.

	Normal Subjects.			Insane Subjects.	
	Number of Observations.	Milligrams Cholesterol per cc. Serum.		Number of Observations.	Milligrams Cholesterol per cc. Serum.
1....	5	2.55	29....	3	1.65
2....	6	1.72	30....	3	1.65
3....	12	1.5	31....	3	1.62
4....	6	1.81	32....	3	1.39
5....	7	2.23	33....	6	1.81
6....	6	1.48	34....	6	2.01
7....	7	1.77	35....	4	1.17
8....	7	1.65	36....	3	1.27
9....	7	1.52	37....	4	2.25
10....	7	1.45	38....	6	2.22
11....	7	1.94	39....	6	1.78
12....	7	1.48	40....	6	2
13....	7	1.65	41....	6	1.52
14....	7	2.22	42....	8	1.47
15....	7	1.55	43....	6	1.65
16....	7	1.62	44....	6	1.27
17....	7	2.03	45....	6	1.39
18....	7	2.68	46....	6	1.97
19....	7	2.35	47....	6	1.73
20....	7	2.18	48....	7	2.91
21....	7	1.96	49....	6	2.97
22....	6	1.50	50....	6	1.78
23....	6	1.52	51....	5	1.60
24....	7	1.36	52....	7	1.23
25....	7	1.86			
26....	6	2.72			
27....	7	1.46			
28....	7	2.66			

The estimates of the cholesterol content of sera obtained from fifty-two persons average 1.82 milligrams to the cubic centimeter of serum, and the range of variation is from 1.17 to 2.97 milligrams.

The average for the twenty-eight normal subjects is 1.87 milligrams, with a range of 1.36 to 2.68 milligrams; and the average for the twenty-four insane subjects is 1.76 milligrams, with a range of 1.17 to 2.97 milligrams.

These observations indicate that the cholesterol content of serum varies very widely among normal persons. Any attempt to determine the pathological significance of cholesterol variations would require first a much larger number of normal specimens of serum than it was possible for us to obtain.

5. Reliability of colorimetric determinations of cholesterol in serum. — The amount of error involved in extracting the cholesterol from 2.5 cubic centimeters of serum may be estimated from the data given in Table I. In the case of Serum 3, the average estimates of the two extracts of untreated serum may be compared. In the other cases it is known that the treated serum contained one milligram of cholesterol to the cubic centimeter in excess of the normal serum, and the difference in the completeness of the two extractions is indicated by the difference between the decimal figures of the average estimates of a given pair. These differences are shown in Table III.

The error involved in the comparison of the tubes is indicated by the variations in the different estimates made upon a given extract, as shown in Table I. If the average of the seven estimates may be accepted as the nearest approach to the actual amount that can be made by this method, all deviations from this average, either above or below, indicate inaccuracies in observation or in the preparation of the solutions observed. The average deviation from the accepted estimate of each extract is shown in Table III.

TABLE III.
(Compiled from Table I.)

	Average Deviation from Average of Seven Observations.	Difference between First and Second Extractions.
Serum 1..... {	.084 .134	} .10
Serum 2..... {	.15 .188	} .02
Serum 3..... {	.083 .04 .084	} .09
Serum 4..... {	.11 .079	} 0
Serum 5..... {	.086 .123	} .13
Serum 6..... {	.129 .121	} .11
Average.....	.108	.075

The errors in extraction are due in large part to the difficulty of handling so small a quantity of serum, and the percentage of error could probably be considerably reduced by using larger quantities.

It is possible that individual estimates of a given extract could be made more accurate by using as the standard a series of cholesterol solutions ranging from .0001 to .0002 gram to one cubic centimeter of chloroform, increasing by .00001 gram. This would require more serum extract for preliminary tests, and for this reason we have not tried it. We have attempted rather to reduce the total error by making several observations upon each extract.

These estimates upon individual sera do not represent uniform accuracy of observation. The sera numbered from 29 to 38 in the table were extracted and observed before we had fully mastered the technic of the method, and the sera numbered from 7 to 16 were tested with chloroform of domestic manufacture. We cannot exclude unsatisfactory

observations without making arbitrary distinctions, and we are unable to obtain material for carrying the research further; it seems best therefore to offer these figures as they are.

SUMMARY.

The yield of cholesterol from quantities of serum as small as two cubic centimeters can be quantitatively estimated by the colorimetric method.

It is not necessary that the cholesterol be extracted in a pure state. By the technic which we recommend it may be obtained sufficiently pure to give the reaction which is characteristic of cholesterol.

Pure cholesterol added to serum can be recovered quantitatively, together with the cholesterol contained in the serum, by the method recommended.

Estimates obtained from fifty-two individual sera range from 1.17 to 2.97 milligrams of cholesterol to one cubic centimeter of serum, the average being 1.82 milligrams. Twenty-four of the sera tested were obtained from insane patients; the estimates made upon these sera show no considerable difference from those made upon sera of mentally normal subjects.

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All communications should be addressed to

HAROLD C. ERNST, M.D.,
Editor of The Journal of Medical Research,
240 Longwood Avenue,
Boston, Massachusetts, U.S.A.

